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Studies on the Activation and Inhibition of Erythrocytic Delta-Aminolevulinic Acid Dehydratase (ALAD)

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STUDIES ON THE ACTIVATION AND INHIBITION OF ERYTHROCYTIC DELTA-AMINOLEVULINIC ACID DEHYDRATASE (ALAD)

by

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VITA

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ABSTRACTS AND PUBLICATIONS

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INTRODUCTION AND REVIEW OF THE RELATED LITERATURE

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INTRODUCTION AND REVIEW OF THE RELATED LITERATURE

A. THE BIOSYNTHESIS OF HEME

All aerobic cells except the normal, mature, non-nucleated erythrocyte synthesize heme, a quadridentate chelate complex of the tetrapyrrole protoporphyrin with Fe^{+2}. Numerous hemoproteins including hemoglobin, myoglobin, catalase, peroxidases, and cytochromes owe their activity, at least in part, to heme. Heme therefore functions as a prosthetic group for oxygen transport, plays an important role in electron transport, and is involved in the detoxification of a variety of xenobiotics. The biosynthetic pathway leading to the formation of heme is the same regardless of its site of synthesis or its ultimate function (FIGURE 1) (Harris and Kellermeyer, 1970).

The synthesis of heme begins with the condensation of the ubiquitous and readily available amino acid glycine and the citric acid cycle intermediate succinyl CoA, with the elimination of carbon dioxide, to form delta-aminolevulinic acid (ALA). This reaction is catalyzed by the mitochondrial enzyme delta-aminolevulinic acid synthetase (ALAS), the only enzyme of heme synthesis requiring a cofactor (pyridoxal phosphate) and to which energy must be

supplied (in the form of activated succinate) (Granick, 1958; Kikuchi et al., 1958). The level of ALAS is the most important rate limiting factor in the heme biosynthetic pathway, being induced by certain sex hormones and drugs (such as barbiturates) and being repressed by heme (Granick, 1966; Granick and Kappas, 1967; Kappas and Granick, 1968). Heme also inhibits the activity of ALAS in a negative feedback fashion (Burnham and Lascelles, 1963; Karibian and London, 1965). Finally, the synthesis of ALAS can be induced by erythropoietin with the subsequent appearance of the other enzymes of heme synthesis (Nakao et al., 1968a).

The substituted monopyrrole precursor of all tetrapyrroles, porphobilinogen (PBG), was first isolated by Westal (1952) from the urine of a porphyric human and characterized by Cookson and Rimington (1953). Dresel and Falk (1953) recognized the possibility that the aromatic compound PBG might be formed by the condensation of two molecules of the aliphatic compound ALA and postulated the existence of an enzyme catalyzing this reaction. This soluble cytoplasmic enzyme delta-aminolevulinic acid dehydratase (ALAD; EC 4.2.1.24), which is present in all aerobic cells including the normal, mature, non-nucleated erythrocyte, was first isolated from ox liver by Gibson and co-workers (1955). These investigators demonstrated that ALAD requires thiol groups for activity and is inhibited in vitro by millimolar concentrations of Pb^{+2}, Ag^{+}, Cu^{+2}, and Hg^{+2}, heavy metals

with a high affinity for sulfhydryl groups (Oehme, 1972). They further demonstrated that ALAD is inhibited *in vitro* by ethylenediaminetetraacetate (EDTA), a chelator of certain divalent metal ions (Martel and Calvin, 1952), strongly suggesting involvement of metal ions in the enzymatic mechanism (Parisi and Vallee, 1969). Heme has been reported to inhibit ALAD in a negative feedback fashion (Calissano *et al.*, 1966).

In the succeeding step of heme synthesis four molecules of PBG condense, under the control of two cytoplasmic enzymes, to form the tetrapyrrole ring compound uroporphyrinogen III. These two enzymes are readily distinguished on the basis of their susceptibility to heat inactivation. PBG deaminase (PD), which is also called uroporphyrinogen I synthetase, is quite heat stable (Bogorad and Granick, 1953) and catalyzes the removal of the amino group from PBG. PD also catalyzes the head-to-tail condensation of four individual pyrroles into the symmetrical uroporphyrinogen I. The second enzyme is uroporphyrinogen isomerase (UI) or uroporphyrinogen III cosynthetase and is quite unstable to heat, being rapidly inactivated at 60°C (Bogorad and Granick, 1953). UI will not, when free of PD, react with PBG or uroporphyrinogen I (Bogorad, 1958). The exact mechanism of the conversion of four molecules of PBG to uroporphyrinogen III by the combined action of PD and UI remains unclear (Gidari and Levere, 1977).

The four acetic acid side chains of uroporphyrinogen III are enzymatically decarboxylated by the action of the soluble cytoplasmic enzyme uroporphyrinogen decarboxylase (UD) to produce coproporphyrinogen III (Mauzerall and Granick, 1958). This enzyme is active only on the fully reduced porphyrinogen (Batlle and Grinstein, 1962). UD decarboxylates the type III isomer at 7.5 times the rate of the type I isomer when present in equal concentrations and requires no cofactor (Cornford, 1964).

The conversion of coproporphyrinogen III to protoporphyrin IX is complex. The propionate groups at positions 2 and 4 of coproporphyrinogen III are decarboxylated and oxidized by the mitochondrial enzyme coproporphyrinogen decarboxylase (CD) resulting in the formation of protoporphyrinogen IX (Granick and Mauzerall, 1958a). This enzyme has no activity toward the coproporphyrinogen I isomer and requires no cofactor (Batlle *et al.*, 1965). Six hydrogens are then enzymatically removed from protoporphyrinogen IX to yield protoporphyrin IX but until recently it was uncertain whether the enzyme catalyzing this reaction is CD or another enzyme (Sano and Granick, 1961). In 1976 Poulson demonstrated that there is indeed a second enzyme specific for protoporphyrinogen IX, protoporphyrinogen IX oxidase (POx).

Heme is formed by the insertion of ferrous, but not ferric, iron into the protoporphyrin IX ring in a reaction

catalyzed by the enzyme heme synthetase (HS) or ferrochelatase (Hasegawa et al., 1970). HS is a mitochondrial enzyme (Jones and Jones, 1969) stimulated by reduced glutathione and inhibited by lead (Goldberg et al., 1956). In addition HS is inducible and subject to end-product inhibition by heme (Hasegawa et al., 1970).

There is a normal physiologic coordination between the synthesis of heme and the synthesis of globin (Morell et al., 1958). It has been demonstrated that, in addition to inhibiting its own formation, heme enhances globin synthesis (Bruns and London, 1965). Furthermore, depletion of available heme results in a suppression of the biosynthesis of globin (Heywood et al., 1972). Thus the self-regulated availability of heme provides the coordination between the synthesis of heme and the synthesis of globin.

B. THE MOLECULAR PROPERTIES OF ALAD

1. THE BASIC PROPERTIES OF ALAD

ALAD isolated from bacterial (Nandi et al., 1968; Van Heyningen and Shemin, 1971; Nandi and Shemin, 1973), plant (Tigier et al., 1970), and mammalian (Doyle, 1971; Gurba et al., 1972; Wilson et al., 1972; Cheh and Neilands, 1973; Wu et al., 1974) sources and purified to varying degrees has been described as having a molecular weight between 240,000 and 290,000. The ALAD of Rhodopseudomonas spheroides (Van Heyningen and Shemin, 1971) and mouse liver (Doyle, 1971) is reported to be composed of six subunits and that from bovine and rat liver of six or seven subunits (Gurba et al., 1972), each with a subunit molecular weight of 40,000. Beef liver ALAD was later reported to contain seven dimeric subunits with molecular weights of 35,500 (Wilson et al., 1972) and seven or eight subunits with molecular weights of 36,000 (Cheh and Neilands, 1973). Wu and associates (1974) found bovine liver ALAD to be composed of eight similar subunits with subunit molecular masses of 35,000; this is consistent with its electron microscopic appearance as discrete lobes arranged at the corners of a cube. Shemin (1976) attributed the discrepancies in the number of reported subunits to the lack of sufficient material from the various sources rather than to actual differences in the enzyme from the various sources.

It is well established that ALAD is inhibited in a negative feedback fashion by heme (Burnham and Lascelles, 1963; Coleman, 1966; Calissano et al., 1966). In addition, the enzyme activity plotted as a function of substrate concentration and of cysteine concentration is sigmoidal (Nandi et al., 1968; Vergnano et al., 1968a). These facts, together with the fact that the enzyme is composed of subunits, suggests that ALAD is an allosteric enzyme (Gerhardt and Pardee, 1962; Martin, 1963; Monod et al., 1963).

ALAD from every source studied has been found to behave like a typical sulfhydryl enzyme (Shemin, 1972). Wilson and fellow workers (1972) demonstrated the presence of 56 cysteine residues per molecule of beef liver ALAD, approximately half of which are involved in inter- or intrasubunit disulfide linkages while the others, as determined by titration with p-chloromercuribenzoate (Erwin and Pedersen, 1968), are free. The report by Shemin (1976) of seven cysteine residues per subunit is in complete agreement with this while the report of Tsukamoto et al. (1975) of nine cysteine residues per subunit is slightly at odds. Both authors report that two or three sulfhydryl groups are readily titrated with (5,5'-dithiobis(2-nitrobenzoic acid)) (Ellman, 1959) only after activation with exogenous thiols or after denaturation of the enzyme, implying involvement in disulfide linkage or existence in a buried position in the enzyme. In the presence of the competitive inhibitor

levulinic acid only one sulfhydryl group can be titrated, implying its presence at the active site and, possibly, the formation of a thiohemiketal between it and the second, non-Schiff base bound ALA molecule. One consequence of the sulfhydryl nature of ALAD is that on purification the sulfhydryl groups become oxidized and the enzyme inactive; the activity of the enzyme can be restored by the addition of thiol activating reagents such as reduced glutathione (GSH), dithiothreitol, and cysteine. In addition, being a sulfhydryl enzyme ALAD is susceptible to inhibition by heavy metal ions which have a high affinity for sulfhydryl groups (Oehme, 1972).

Crude preparations of mouse liver ALAD have been reported to be activated by the *in vitro* addition of EDTA while EDTA has no effect on the purified enzyme (Coleman, 1966). ALAD from *R. spheroides* and *R. capsulata* is similarly unaffected by EDTA (Nandi and Shemin, 1973). *In vitro* EDTA, however, does inhibit ALAD from all other reported bacterial (Yamasaki and Moriyama, 1971), fungal (Komai and Neilands, 1969), yeast (DeBarreiro, 1967), plant (Nandi and Waygood, 1967; Shetty and Miller, 1969; Tigier *et al.*, 1970; Schneider, 1970), and animal (Gurba *et al.*, 1972; Wilson *et al.*, 1972; Yamada, 1972; Border *et al.*, 1976a) sources, strongly suggesting the involvement of divalent metal ions in the mechanism of the ALAD from these sources (Parisi and Vallee, 1969).

2. THE EFFECT OF METAL IONS ON ALAD ACTIVITY

It appears that ALAD, regardless of source, has the same molecular mass, number of subunits, sulfhydryl character, metal ion requirement for maximal activity, and allosteric nature. The exact nature of the effect of metal ions, with the exception of lead (which will be discussed further in other sections), on ALAD activity *in vitro* and *in vivo* seems, however, to depend on the source of the enzyme and even then there is often disagreement.

Komai and Neilands (1968) found that ALAD activity in crude extracts of the smut fungus *Ustilago sphaerogena* grown in its sporoidal stage depends on the concentration of Zn^{+2} in the culture media. Enzyme activity is not lost on dialysis of the crude extracts and *in vitro* addition of Zn^{+2} has no effect on the enzyme up to 10^{-4} M when it is inhibitory. They, therefore, concluded that Zn^{+2} is necessary for the synthesis of ALAD at the translation level but did not preclude the possible presence of a firmly bound essential metal ion since Zn^{+2}, Fe^{+2}, and, to a lesser extent, Cd^{+2} enhance the activity of the purified enzyme as well as that treated with chelating agents. In a subsequent paper (1969) they reported that Cu^{+} chelating agents are powerful inhibitors of the enzyme and there is a moderately good correlation between the activity of ALAD from *U. sphaerogena* and its copper content after purification. The ligand-treated enzyme from *U. sphaerogena* cannot be reconstituted by Cu^{+2}

because it is a potent inhibitor but it can be reactivated by Zn^{+2} which is isoelectronic with Cu^{+}.

ALAD from the yeast Saccharomyces cervisiae is also inhibited by millimolar concentrations of Cu^{+2} as well as by Hg^{+2} while Mg^{+2} is added to the enzyme preparation without effect (DeBarreiro, 1967). Divalent magnesium at a concentration of 3.3 mM is, however, necessary for the maximum activity of the ALAD from Mycobacterium phlei. At a concentration 0.33 mM, Mg^{+2} can reverse the EDTA-induced inhibition of the enzyme from this source (Yamasaki and Moriyama, 1971).

ALAD from the photosynthetic bacteria R. spheroides and R. capsulata is the only ALAD unaffected by EDTA. A purified and well-dialyzed preparation of ALAD from R. capsulata is active without the addition of any cation but millimolar concentrations of either Fe^{+2} or Fe^{+3} inhibit the enzyme (Nandi and Shemin, 1973). The enzyme from R. spheroides requires K^{+} or a related ion such as Li^{+}, Rb^{+}, NH_4^{+}, or Mg^{+2} for activation at low substrate concentrations (Nandi et al., 1968). In the presence of these ions the enzyme associates into a mixture of monomer, dimer, and trimer (Nandi and Shemin, 1968a).

Wheat leaf ALAD is completely inhibited by millimolar concentrations of Cu^{+2} and Hg^{+2} while the same concentration of Na^{+}, K^{+}, Fe^{+2}, Co^{+2}, Ni^{+2}, Zn^{+2}, and Pb^{+2} is without effect on the enzyme. A 10^{-4} M concentration of Mg^{+2}, Ca^{+2},

or Mn^{+2} can activate wheat leaf ALAD, the activation by Mn^{+2} being able to overcome inhibition by EDTA in a stoichiometric fashion (Nandi and Waygood, 1967). Tobacco leaf ALAD is inhibited by low concentrations of both Fe^{+2} and EDTA; low concentrations of both Mg^{+2} and Mn^{+2} partially reverse the inhibition of ALAD by EDTA but at higher concentrations only Mg^{+2} is an activator (Shetty and Miller, 1969). It has been reported by Schneider (1970) that ALAD from spinach leaves is inactive after dialysis and application of EDTA but is reactivated by Mg^{+2} and, to a lesser degree, Mn^{+2}; low concentrations of Ca^{+2}, Fe^{+2}, and Zn^{+2} inhibit the enzyme from this source but Na^{+}, K^{+}, and NH_4^{+} are without effect. In contrast to this, Tigier et al. (1970) reported the maximum activity of ALAD from the soybean callus is observed when it is grown is a culture medium containing 10^{-6} M Zn^{+2} and concluded Zn^{+2} is essential for enzyme synthesis.

The divalent cations Mg^{+2}, Ca^{+2}, Ba^{+2}, Mn^{+2}, Fe^{+2}, and Zn^{+2} have no effect on mouse liver ALAD while Cu^{+2} and Pb^{+2} inhibit the enzyme at low concentrations. ALAD from this source is moderately activated by Fe^{+2} at 10^{-3} M and more markedly activated by Hg^{+2} at 10^{-4} to 10^{-3} M which can also prevent the inhibition of ALAD by 10^{-5} M Cu^{+2} (Coleman, 1966).

Tomio and co-investigators (1968) found that neither purified ALAD nor the crude preparation from the rat Harderian gland requires the addition of any metal ion for maximal

activity. However, Cu^{+2}, Hg^{+2}, Ag^{+}, and Pb^{+2}, each at a final concentration of 0.1 to 1.0 mM, inhibit the enzyme. While millimolar concentrations of $MgCl_2$ have a moderate inhibitory effect on the enzyme, when Mg^{+2} is added as the sulfate it activates the enzyme as do the sulfate salts of NH_4^{+} and K^{+}.

Rat erythrocyte ALAD has been reported to be activated to 250% of control activity by 2 mM Al^{+3}, to 240% of control activity by 0.18 mM Zn^{+2}, and to 360% of control activity by the combination of the two (Meredith et al., 1974). Meredith and associates also report that aluminum sulfate activates rat liver ALAD in vivo. Zinc also affects erythrocytic ALAD in vivo for its activity is markedly decreased when the rats are fed a low zinc diet. The in vitro addition of Zn^{+2} to hemolysates of Zn^{+2} deficient rats results in only a slight increase in ALAD activity, suggesting Zn^{+2} is required at the level of ALAD synthesis (Finelli et al., 1974; Finelli et al., 1975).

In an early report of the metal content of ALAD purified from beef liver, Iodice et al. (1958) found 0.1% copper in the enzyme with magnesium being the only other metal present. Gurba and associates (1972), on the other hand, reported the hepatic ALAD to contain approximately 1 g-atom of zinc per 270,000 g of protein as well as smaller, non-stoichiometric amounts of iron and cobalt.

According to Cheh and Neilands (1973) the ALAD from

bovine liver contains 1.2 g-atoms of zinc per mole of enzyme and possesses only 25% of the activity ultimately observed after titration with between 5 and 6 g-atoms of Zn^{+2} per mole. This represents the upper limit of the Zn^{+2} requirement because kinetic and competitive factors diminish the effectiveness of added metal hence only half of the 7 or 8 subunits of the enzyme must be involved in Zn^{+2} binding. These investigators also reported that Cd^{+2} restores full activity to the purified enzyme but Mg^{+2}, Ca^{+2}, Cr^{+3}, Fe^{+2}, Fe^{+3}, Ni^{+2}, Cu^{+}, and Cu^{+2} are inactive. Shemin (1976) similarly found, by determining the number of atoms of Zn^{+2} per mole of enzyme necessary to restore full activity to the bovine apo-enzyme, that the enzyme contains 4 to 6 atoms of zinc per molecule of enzyme. It was further discovered that the addition of Zn^{+2} is ineffective in restoring activity to the purified enzyme unless it is first reduced by a reduced thiol.

The activity of beef liver ALAD inhibited by 10 μM EDTA can be completely restored by 100 μM Zn^{+2} which is considered by Wilson et al. (1972) to be an absolute requirement for the enzyme. In addition, they reported that 1 mM Zn^{+2} is a competitive inhibitor of ALAD, 100 μM Mg^{+2}, Ca^{+2}, Fe^{+2}, and Cu^{+2} have no effect on the enzyme, and 100 μM Cd^{+2} is unique in that at low substrate concentrations it inhibits ALAD while at higher substrate concentrations it activates the enzyme. In a rather unusual study Hamp

and Kriebitzsch (1975) found that both 500 μM Zn^{+2} and 100 μM Cd^{+2} enhance the activity of bovine erythrocytic ALAD when stored with the enzyme for two to six weeks at -30°C.

The effect of various metal ions on purified human erythrocytic ALAD was investigated by Calissano et al. (1965) who found K^{+}, Mg^{+2}, Ca^{+2}, and Fe^{+3} to be without effect on the enzyme while Mn^{+2} and Co^{+2} are weak and Pb^{+2}, Ag^{+}, Cu^{+2}, and Hg^{+2} are powerful inhibitors of the enzyme. On the other hand, Fe^{+2} stimulates ALAD at all concentrations from 5×10^{-6} M to 5×10^{-3} M and Zn^{+2} stimulates ALAD at all concentrations between 5×10^{-5} M and 5×10^{-4} M while inhibiting it at 5×10^{-3} M. According to Collier (1971) a fresh hemolysate dialyzed against a solution of EDTA has only 10% of the activity of an undialyzed preparation and its activity can only be restored by Mn^{+2}. Millimolar concentrations of Fe^{+2} and Co^{+2} inhibit the undialyzed enzyme, Ca^{+2} has no effect on it, and Mn^{+2} activates it.

Abdulla and Haeger-Aronsen (1971) incubated various concentrations of Zn^{+2} with a human whole blood hemolysate for four hours and found that 4.6×10^{-5} M Zn^{+2} inhibits ALAD slightly while 7.6×10^{-4} M and 1.5×10^{-3} M Zn^{+2} stimulate the enzyme to 172 and 201% of the control activity, respectively. These investigators also reported the oral administration of 30 to 40 mg of Zn^{+2} (as the sulfate) per day for four weeks in one volunteer resulted in a successive increase in erythrocytic ALAD activity but they

presented no actual data for this.

The effects of Mg^{+2}, Ca^{+2}, Mn^{+2}, Co^{+2}, Ni^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2} at concentrations ranging from 10^{-7} M to 10^{-2} M on the ALAD activity of human whole blood hemolysates was investigated by Mitchell and associates (1977). They discovered that both Cd^{+2} and Hg^{+2} stimulate ALAD at 10^{-5} M before inhibiting it at higher concentrations, Zn^{+2} stimulates the enzyme maximally at 10^{-4} M but at no concentration is it inhibitory, and the other ions are without effect. They also found that Zn^{+2} can protect ALAD against inhibition by EDTA down to 10^{-1} M EDTA and that the stimulation of the enzyme by Zn^{+2} is additive with that by sulfhydryl reagents, implying different mechanisms of stimulation.

The inhibition of ALAD by the in vitro addition of EDTA is well established but its in vivo effect has only recently been studied. At a plasma EDTA concentration of 0.2 to 0.4 mM a 5% decrease in the erythrocytic ALAD activity and a 14% decrease in the hepatic ALAD activity of the normal rat is observed while at the same plasma concentrations in vivo lead-induced inhibition of hepatic ALAD is almost completely reversed (Hammond, 1973). These effects of EDTA on ALAD in vivo apparently also occur in man. In lead-intoxicated children treated with Na_2(Ca-EDTA) there is a transient increase in serum and urinary ALA concentrations (possibly reflecting a further inhibition of the enzyme by

EDTA) followed by a rapid and pronounced fall in both serum and urinary ALA concentrations (reflecting removal of the lead from, hence reactivation of, ALAD) (Chisolm, 1968a).

Trisodium calcium diethylenetriaminepentaacetate, Na_3(Ca-DTPA), the most effective and widely used chelation agent for reducing actinide contamination in man, has an ability to remove zinc that is approximately 100 times more than that of Na_2(Ca-EDTA) (Catsch et al., 1968). In baboons treated with Na_3(Ca-DTPA), whenever the concentration of Zn^{+2} in the catheterized urine was greater than approximately four times the normal value erythrocytic ALAD activity was decreased to less than one-half of the normal value (Cohen and Guilmette, 1976). In patients with cirrhosis a similar effect is observed for in these patients a 40% decrease in plasma zinc concentrations and a 48% decrease in erythrocytic ALAD activity has been observed. The fact that the activity of ALAD in patients with cirrhosis is less enhanced by the in vitro addition of Zn^{+2} than it is in lead intoxicated patients led these investigators to conclude there is a decreased synthesis of ALAD as a result of the decrease in Zn^{+2} (Allain et al., 1977).

Copper deficiency may not have the same effect on erythrocytic ALAD activity zinc deficiency has. In a single patient receiving intravenous feedings containing insufficient copper, a copper deficiency anemia resulted but the erythrocytic ALAD activity was normal. This is consistent

with the observation of the same researchers that the erythrocytic ALAD activity in rats fed a copper deficient diet containing adequate levels of zinc is normal (Finelli *et al.*, 1974). This, however, is in marked contrast to the observation that hepatic ALAD activity in rats and erythrocytic ALAD activity in ducklings is decreased 30 to 50% if dietary copper is missing (Iodice *et al.*, 1958).

There are three heavy metals that are generally recognized as being ubiquitous environmental contaminants: lead, mercury, and cadmium (Vallee and Ulmer, 1972). The *in vivo* effect of each of these on human erythrocytic ALAD activity has been investigated. Wada *et al.* (1969) found a moderate decrease in the ALAD activity of workers occupationally exposed to mercury below the old threshold limit value (TLV) of 0.1 mg/m^3 whose urinary mercury exceeded 200 µg/gm creatinine; neither ALAD activity nor urinary mercury levels, however, correlated with the erythrocyte glutathione levels or the urinary ALA excretion, which were normal in all cases. Among workers exposed to mercury vapor below the new TLV of 0.05 mg/m^3 and at the exposure level of the general population there is no decrease in erythrocytic ALAD activity or increase in urinary ALA (Lauwerys and Buchet, 1973). This same group of investigators also found that occupational exposure to cadmium has no effect on ALAD activity, urinary ALA excretion, or red cell glutathione levels (Lauwerys *et al.*, 1973; Roels *et*

al., 1975). Thus of the three metals generally recognized as environmental contaminants only lead causes a significant in vivo inhibition of erythrocytic ALAD.

It is interesting to note that Sn^{+2}, which has the same electronic configuration as Pb^{+2}, inhibits erythrocytic ALAD activity when administered intraveneously to rabbits but Sn^{+4} does not (Chiba and Kikuchi, 1978).

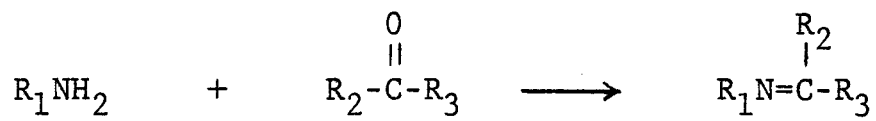
Thus, while there is no universal agreement with respect to the metal ion requirement of ALAD, there does appear to be nearly unanimous agreement that the enzyme loses activity on purification. This appears to be due to the tendency of ALAD to lose its metal ion on purification or to the oxidation of its many sulfhydryl groups on purification or to both.

While there is no agreement about the metal ion requirement of ALAD it is, nonetheless, tempting to speculate that: the enzyme from anaerobic microbial sources which synthesize the cobalt-containing porphyrin vitamin B_{12} requires Cu^{+}, Zn^{+2}, or Mg^{+2}; that from photosynthetic microorganisms which synthesize the manganese-containing porphyrin chlorophyll requires Ca^{+2}, Mg^{+2}, or Mn^{+2}; ALAD from eukaryotic microorganisms and mammalian sources which synthesize the iron-containing porphyrin heme require Cu^{+}, Zn^{+2}, or Fe^{+2}. However, while on the basis of the current evidence there is considerable overlap of metal ion requirements for ALAD but no consensus, the generalizations with

respect to the metal ion requirement of ALAD based on the ultimate destination of the porphyrin moiety appears unjustified. Clearly, further work is necessary to clarify the exact nature of the metal ion requirement of ALAD from the various sources.

3. THE MECHANISM OF PORPHOBILINOGEN SYNTHESIS

A Schiff base is an imine resulting from the condensation of an amine and a carbonyl-containing compound such as an aldehyde or a ketone (Snell and DiMari, 1970):



Incubation of an enzyme with the substrate in the presence of sodium borohydride ($NaBH_4$) with the consequent loss of enzymatic activity confirms the formation of a Schiff base between the carbonyl group of the substrate and the epsilon-amino group of a lysyl residue of the enzyme. Concomitant with the loss of enzymatic activity is a stoichiometric binding of radioactive substrate to the enzyme (Horecker et al., 1963; Grazi et al., 1963).

ALAD from R. spheroides (Nandi and Shemin, 1968b; Van Heyningen and Shemin, 1971), R. capsulata (Nandi and Shemin, 1973), mouse liver (Doyle, 1971) and bovine and rat liver (Gurba et al., 1972) is almost completely inactivated by $NaBH_4$ in the presence of ^{14}C ALA with radioactivity bound

to the enzyme in a stoichiometric ratio. The formation of a Schiff base by one molecule of ALA with ALAD precludes the initial formation of a Schiff base between the two substrate molecules. Furthermore, the formation of a Schiff base between ALA and ALAD results in a stabilized carbanion which can make a nucleophilic attack onto the carbonyl carbon of a second molecule of ALA resulting in an aldol condensation. There is then an enzyme catalyzed elimination of water between the carbon atom bearing the newly formed hydroxyl group and the adjacent carbon atom. In the final step the amino group of the ALA molecule not in Schiff base linkage with the enzyme makes a nucleophilic attack on the highly reactive carbon atom in Schiff base linkage with the enzyme resulting in the formation of a gem diamine. The amino group of the enzyme then leaves the gem diamine and the conjugated pyrrole PBG, which is no longer able to react with the enzyme, is formed. The formation of PBG in this manner (i.e. transamination) serves two purposes; it is a trap driving the reaction to completion and it provides a mechanism for freeing the product from the active site of the enzyme (Nandi and Shemin, 1968b).

In an extension of this mechanism Cheh and Neilands (1974) proposed the participation of divalent zinc in a Lewis acid polarization of the carbonyl group of the second molecule of ALA, facilitating the nucleophilic attack of the first, Schiff base-bound, substrate molecule.

The observation that only four of the eight subunits of ALAD form a Schiff base with ALA indicates the enzyme exhibits the phenomenon of negative cooperativity and half-site reactivity (Shemin, 1976). That is, the maximal stoichiometric yield of an enzyme-substrate covalent intermediate or product in a single turnover is equal to half the number of apparently equivalent active sites. This is a consequence of the peculiar quaternary structure of the native enzyme or the native enzyme-substrate intermediate resulting in the absence of a twofold axis of symmetry in the oligomeric state (Seydoux et al., 1974).

Batlle and co-workers (1978) investigated the dissociation, reassociation, and rehybridization of bovine liver ALAD attached to Sepharose. They found that each catalytic site of the enzyme could function independently of the other within a dimeric formation. They, therefore, postulated (Batlle and Stella, 1978) that the minimum structure necessary for enzymatic activity is a dimer having one subunit involved in the formation of a Schiff base with one molecule of ALA while the second subunit binds noncovalently with the second molecule of ALA, holding it in place. The histidine residue present in each of the active sites (Tsukamoto et al., 1975) are, in this model, assigned the role of transferring protons to and from the active center of the basic mechanism described above. The two subunits are held together, according to this model, at intersubunit

contact sites by ionic, covalent, coordinate covalent, or hydrogen bonds in which the sulfhydryl groups of the enzyme as well as the metal activator (e.g. divalent zinc) participate.

The half site reactivity of a functional dimer catalyzing this rather rare reaction in which two identical substrate molecules participate would help explain the early observation of Granick and Mauzerall (1958b) that the affinity of ALAD for the first molecule of ALA is at least ten times greater than its affinity for the second. The first molecule binds covalently to one subunit while the second molecule binds noncovalently to the second subunit.

4. THE ACTIVATION OF LEAD-INHIBITED ALAD BY THIOLS, METAL IONS, pH CHANGE, AND HEATING

Lead inhibits ALAD from all mammalian sources *in vivo* and *in vitro* in a noncompetitive fashion (Wilson *et al.*, 1972; Sassa *et al.*, 1975). The inhibition of ALAD can, nonetheless, be reversed both *in vitro* and *in vivo*.

The *in vivo* and *in vitro* inhibition of erythrocytic ALAD caused by lead can be reversed by the *in vitro* addition of thiol compounds including reduced glutathione (Lichtman and Feldman, 1963), cysteine (Bonsignore *et al.*, 1965), and dithiothreitol (Granick *et al.*, 1973). The reactivation of the lead inhibited ALAD by thiols is considered to be the result of a thiol-mercaptide exchange between the added

thiol and the lead-sulfhydryl complex of the lead inhibited enzyme. Hapke and Prigge (1973) attributed the difference in thiol activated ALAD between a lead contaminated group and a control group to a normalization of the free part of the enzyme by a resynthesis of the enzyme.

Granick and fellow investigators (1973) found an excellent correlation between blood lead levels and the ratio of thiol activated ALAD activity to nonactivated ALAD activity. The linearity of this ratio, which eliminates the broad distribution of ALAD activities due to genetic variation (Sassa et al., 1975), down to unity at a blood lead level of 15 μg per 100 ml of whole blood led these workers to conclude that this is the threshold for the effect of lead on ALAD. Below this concentration they assume lead binds to some group (perhaps on the erythrocyte membrane) with a higher affinity for lead than ALAD.

These results have led some to hypothesize that the endogenous concentration of GSH in erythrocytes plays a regulatory role in the activity of ALAD (Moore et al., 1971) and that this control mechanism might be affected by lead (DeBarreiro, 1969). Indeed, there is a well established moderate but significant decrease in the endogenous erythrocyte GSH concentration in lead intoxicated workers (Shiraishi, 1952; Rubino et al., 1963; Batolska and Marinova, 1970). Furthermore, one group reports that the in vivo administration of GSH to lead intoxicated workers improved

the decreased ALAD to some extent (Nakao et al., 1968b).

Abdulla and Haeger-Aronsen (1973) observed that relatively high concentrations of zinc could antagonize the heavy metal-induced inhibition of erythrocytic ALAD in vitro. Finelli and fellow workers (1975) have reported the reversal of the in vivo and in vitro lead-induced inhibition of erythrocytic ALAD by the in vitro addition of divalent zinc. These authors also noted a protective effect of zinc on the inhibitory effect of lead during in vivo studies on rats. They suggested the in vivo stimulation of lead inhibited ALAD by zinc can be a useful tool for the diagnosis of lead intoxication. Their conclusion, on the basis of these results, was that zinc is functioning at the active site of ALAD, possibly at sulfhydryl groups, to displace lead and thereby reactivate the enzyme.

In 1976 Haust and associates reported the case of a 7 year old lead poisoned girl with pica who had inadvertently ingested approximately 5 g of zinc oxide. These workers noted a tenfold increase in erythrocytic ALAD activity on day 5 after the ingestion and another, less dramatic, rise on day 13 after the ingestion of zinc and another on day 17. These increases in erythrocytic ALAD activity were related to increases in red cell zinc provided there was no concomitant increase in red cell lead or copper.

Aluminum has been reported to activate in vitro lead inhibited erythrocytic ALAD in vitro and hepatic ALAD

activity *in vivo* (Meredith *et al.*, 1974). These observations, however, should be viewed with caution since these authors used a 0.5 M potassium phosphate buffer which might interfere with the results (Finelli et al., 1974).

Mitchell *et al.* (1977) used an assay incorporating both divalent zinc and a thiol compound, GSH, with incubation with the substrate at 50°C for 10 to 20 minutes. The extent of activation of normal erythrocytic ALAD by zinc and by GSH was found to be similar but the combined effects were additive, suggesting these two agents activate ALAD by different mechanisms. The natural logarithm of the ratio of activated erythrocytic ALAD activity to nonactivated erythrocytic ALAD activity was found to be directly proportional to the blood lead concentration with the correlation between these two values being higher than that reported by Granick and co-workers (1973) for their GSH activated system as reported above.

Nikkanen and associates (1972) have demonstrated that the optimum pH for erythrocytic ALAD determination is lower in persons occupationally exposed to lead than in normal individuals. Using this observation, Tomokuni (1974) found the rate of decrease of the ratio of erythrocytic ALAD activity determined at pH 6.8 to that determined at pH 6.0 to be proportional to the increase of the blood lead level. From these results Tomokuni concluded that lead regulates ALAD activity from an allosteric site on the molecule which

changes in conformation as the pH is lowered, thereby preventing lead from interfering with the active site of the enzyme.

ALAD is remarkably heat stable, being capable of withstanding temperatures as high as 60°C for at least 20 minutes without significant loss of activity (Coleman, 1966; Nandi et al., 1968; Wilson et al., 1972). The reaction rate increases nearly linearly with increasing temperature up to 60°C with the reaction rate at 50°C being nearly three times that at 37°C (Mitchell et al., 1977). The thermal stability of ALAD can be exploited to determine whether or not ALAD is a regulatory enzyme because mild heating is classically employed to make such enzymes insensitive to metabolite inhibition at allosteric sites (Gerhardt and Pardee, 1962; Martin, 1963; Monod et al., 1963).

Several groups of investigators have used the technique of heating the whole blood hemolysate at 60°C for 5 minutes prior to incubation with substrate in order to determine the mechanism by which lead inhibits ALAD with mixed success and contradictory conclusions (Bonsignore et al., 1968; Vergnano et al., 1968b; Tomokuni and Kawanishi, 1975; Chiba, 1976). There was complete agreement that the heat treatment reversed the inhibitory effect of lead on erythrocytic ALAD if the inhibition was due to chronic in vivo exposure to lead. There was similar agreement that the heat treatment potentiated lead-induced inhibition if

lead was added to a hemolysate of normal blood prior to preincubation and weakened the inhibitory action of lead if it was added to a normal blood hemolysate after preincubation. On the other hand, the ALAD activity of a liver homogenate was actually decreased by the heat treatment (Coleman, 1966; Vergnano et al., 1968b). These results led Vergnano et al. (1968b) and Tomokuni and Kawanishi (1975) to conclude that lead causes an allosteric modification of the active site resulting in enzymatic inhibition. Bonsignore et al., (1968) concluded, on the basis of the same results, that the lead-induced inhibition of ALAD is due to the action of a thermolabile allosteric effector or to a structural modification at the level of synthesis of the enzyme or to the variation in the level of a phantom normal isoenzyme. Chiba (1976) hypothesizes the existence of two components of erythrocytic ALAD: type 1, inherently active without heat treatment, is inhibited by lead in a mannner that cannot be restored by heat treatment; type 2, inherently inactive without heat treatment and relatively insensitive to lead, may be inactivated by higher concentrations of lead. The explanation offered by Chiba appears to be the most reasonable especially if one imagines the type 2 ALAD to be that fraction of all ALAD inhibited in a negative feedback fashion by heme (Calissano et al., 1966), the level of which could be increased in chronic lead intoxication as a result of hemolysis (Hernberg et al., 1967; Berk et al.,

1970). Indeed both the degree of hemolysis (Hernberg et al., 1967) and the ratio of heat treated erythrocytic ALAD activity to unheated erythrocytic ALAD activity (Tomokuni and Kawanishi, 1975; Chiba, 1976) are directly proportional to the blood lead level.

The basic conclusion derived from the reactivation of lead-inhibited erythrocytic ALAD by thiols, metal ions, pH change, and heating is that lead inhibits the enzymatic activity directly and does not inhibit the synthesis of the enzyme. What is still open to question is whether this inhibition is at the active site or at an allosteric site.

In addition, the assessment of erythrocytic ALAD activity with and without activation of the enzyme by thiols, metal ions, pH change, or heating and the calculation of the simple ratio of activated to nonactivated ALAD activity has several advantages over the use of the nonactivated ALAD assay in the assessment of lead intoxication. These advantages have been summarized by Mitchell and associates (1977) and include:

a. the ratio increases with an increase in the blood lead level and is more closely correlated with it than is the erythrocytic ALAD activity alone
b. the units of enzyme activity need not be computed thus avoiding the confusion of the many different methods of expressing the erythrocytic ALAD activity
c. the results do not need to be normalized by use of

the hemoglobin concentration or the hematocrit

d. the broad range of enzymatic activity resulting from genetic differences in erythrocytic ALAD activity is eliminated as a source of error

e. activation of the enzyme provides a positive internal control of the test.

C. THE ACTIVITY OF ALAD IN DISEASE STATES

1. THE ACTIVITY OF ALAD IN CERTAIN ANEMIAS

The components of the heme biosynthetic pathway in the erythrocyte are sensitive to changes in red cell turnover and red cell age in both health and disease as well as to a variety of genetic, developmental, and environmental factors (Harris and Kellermeyer, 1970) and ALAD is no exception. For example, human erythrocytic ALAD is inherited in a co-dominant fashion resulting in a broad range of enzymatic activity (Sassa et al., 1973a). As additional examples, repeated phlebotomy (Abdulla et al., 1975), phenylhydrazine-induced (i.e. hemolytic) anemia (Abdulla and Svensson, 1978), and iron deficiency anemia (Sharma, 1973) in rats stimulate erythropoiesis and a subsequent increase in ALAD activity which can be correlated with a greater number of circulating reticulocytes. It is, therefore, not surprising that changes in erythrocytic ALAD activity in a variety of human anemias, both before and after the initiation of therapy, have been reported. These reports, however, have not always been in agreement.

A hemolytic disease exists in any situation in which the in vivo life span of the erythrocyte is shorter than normal. The hemolytic process is considered to be compensated if the rate of red cell destruction is such that an increase in erythrocyte production can compensate for the

excessive destruction. A compensated hemolytic process is generally reflected by a partial shift of reticulocytes from the bone marrow to the peripheral blood. In severe, albeit at least partially compensated, hemolytic anemias due to hemoglobinopathies (including SS, SC, and S-β-thalassemia) and other, miscellaneous, causes (e.g. autoimmune hemolytic anemia) as well as in compensated mild hemolysis (e.g. β-thalassemia trait) ALAD activity is significantly increased. In addition, the increase in erythrocytic ALAD activity in these hemolytic diseases is clearly associated with an increase in the number of reticulocytes (Rubino et al., 1960; Feldman and Lichtman, 1964; Anderson et al., 1977).

The megaloblastic anemias are due to varying degrees of abnormality in the metabolism of vitamin B_{12} or folic acid or both together. The result of this abnormal metabolism is ineffective erythropoiesis producing, if untreated, an uncompensated macrocytic, hypochromic anemia. In the megaloblastic anemias due to vitamin B_{12} or folic acid deficiencies erythrocytic ALAD activity is reported to be both decreased (Rubino et al., 1960) and increased with no substantial difference in technique hence no ready explanation for the discrepency in the results (Battistini et al., 1971). Both authors, however, agree that the activity of erythrocytic ALAD increases with the reticulocyte response during the response to the appropriate therapy.

Iron lack or deficiency anemia is an uncompensated

hypochromic microcytic anemia due to depletion of the iron normally available to meet metabolic requirements. In iron deficiency anemia erythrocytic ALAD activity has been reported to be unchanged (Rubino et al., 1960; Lichtman and Feldman, 1963; Prato et al., 1968; Gutniak et al., 1971; Battistini et al., 1971) and increased (Heilmeyer, 1964; Kaneko, 1970; Mazza et al., 1971; Chalevelakis et al., 1977). The explanation of this difference is suggested by the observation of Sharma (1973) that the enzyme activity in iron deficient rats was unchanged when expressed as units/ml of blood but was increased when more properly expressed as units/ml of packed red cells. There is, nonetheless, agreement that the activity of erythrocytic ALAD increases with the reticulocyte response during therapy (Rubino et al., 1960; Mazza et al., 1971).

The association of an increase in erythrocytic ALAD activity with the reticulocytosis of hemolytic anemia as well as with that associated with the response of vitamin B_{12}, folic acid, and iron deficiency anemias to appropriate therapy has an important implication. A decrease in the components of the heme biosynthetic pathway with red cell ageing is anticipated because hemoglobin synthesis is active in bone marrow erythroid cells and reticulocytes but not in circulating mature erythrocytes that have lost their cellular organelles. These observations suggest that with the maturation of the erythrocyte, in addition to the loss of

mitochondrial heme biosynthetic enzymes, there is a more gradual decline in the activity of the soluble cytoplasmic enzyme ALAD. The decline in enzymatic activity could reflect a degradation of enzyme protein or another mechanism such as the loss of a cofactor or the progressive increase in the concentration of an inhibitor. To evaluate the mechanism of the decline in ALAD activity with an increase in the age of the erythrocyte population it might be important to have an experimental model in which there is little genetic variation in enzymatic activity but varying degrees of a naturally occurring reticulocytosis.

2. AN ANIMAL MODEL FOR STUDYING THE ASSOCIATION OF ERYTHROCYTIC ALAD ACTIVITY WITH RETICULOCYTOSIS

The neonatal and developing rat is a potentially useful model for studying the relationship between erythrocytic ALAD activity and the degree of reticulocytosis. A macrocytic hypochromic anemia is characteristic of the early neonatal period of the rat. This anemia appears to be a partially compensated hemolytic anemia in light of the erythroid hyperplasia, the increased rate of macrocyte disappearance, and the increased degree of reticulocytosis (ranging from 89% at day 1 to 29% at day 10 and 3.7% at day 40) that has been observed (Lucarelli et al., 1964). These unique features had initially suggested that erythropoietic activity in the neonatal rat is governed by a mechanism

different than that of the adult (Lucarelli et al., 1968). It has, however, subsequently been demonstrated that erythropoiesis in the neonatal rat is indeed regulated by the same hypoxia-erythropoietin mechanism present in the adult (Matoth and Zaizov, 1970; Zaizov and Matoth, 1970). The unique hematologic picture presented by the newborn and developing rat is a reflection of the transition from hepatic to bone marrow erythropoiesis, a transition similar in many ways to that observed during the last trimester of normal human gestation (Michels, 1931).

A preliminary observation that adult rats weighing 200 g or more had approximately one-half of the erythrocytic ALAD activity of younger animals weighing 100 g or less (Finelli et al., 1974) suggests that there may indeed be a correlation between erythrocytic ALAD activity and reticulocyte percent in the developing rat. If that were indeed the case, the developing rat could provide a useful model for investigating the mechanism of the decline of erythrocytic ALAD activity with an increase in the age of the cell. In addition, it could serve to demonstrate an important source of variability in lead poisoning analysis in newborn and developing rats.

3. THE ACTIVITY OF ALAD IN LEAD POISONING ANEMIA

The important hematologic finding in lead poisoning is anemia which is perhaps the best understood effect of lead

in man. A decrease in the blood hemoglobin concentration begins to appear at relatively low levels of lead exposure; the ultimate anemia can be constant and severe in children (Watson et al., 1958) but is generally mild in the adult (Rubino et al., 1959; Griggs, 1964; Waldron, 1966) except at very high levels of exposure when a frank anemia is observed (Williams, 1966). The threshold of blood lead exposure for the appearance of anemia appears to be a blood lead level of 40 μg/100 ml in children (Pueschel et al., 1972; Betts et al., 1973) and 50 μg/100 ml in adults (Tola et al., 1973). The anemia is typically normocytic and slightly hypochromic (Fullerton, 1952) and the reticulocyte percent is usually elevated to the 2 to 7% range. The mild anemia found in most patients with lead poisoning appears to be the result of several different effects of lead.

Lead has a direct, hemolytic action on the erythrocyte resulting in a moderate shortening of the erythrocyte survival time that can be correlated with anemia, reticulocytosis, and blood lead levels (Hernberg et al., 1967). These authors found the erythrocyte destruction to be nonrandom, indicating lead shortens the true life span of the erythrocyte, but others found a random shortening of the red cell life span (Berk et al., 1970). The shortening of the red cell life span is of such a degree that the bone marrow should be able to adequately compensate and hemoglobin depression should not be observed. That hemoglobin

depression is observed suggests that lead also inhibits the production of erythrocytes by the bone marrow.

There is indeed direct evidence for lead inhibition of both heme and globin biosynthesis. In acute lead poisoning the synthesis of globin has been demonstrated to be markedly disturbed although the investigators were unable to determine whether or not this was primarily due to lead acting at some level in globin synthesis (White and Harvey, 1972). It was subsequently demonstrated that the abnormality in the synthesis of globin seen in lead poisoning occurs only secondarily to a deficit in the production of heme (Piddington and White, 1974). The deficit in heme production is the consequence of the inhibition of the two heme biosynthetic enzymes that are sensitive to lead.

Gibson and Goldberg (1970) measured heme synthetase activity of brain, liver, kidney, and bone marrow in rats exposed subacutely to parenteral lead and found no inhibition by lead. They did, however, find that the addition of lead acetate in concentrations similar to those present in the original unhomogenized tissues, 10^{-4} M, produced marked inhibition of HS. A direct consequence of HS inhibition *in vivo* is an increase in the concentration of protoporphyrin IX in the blood (generally referred to as free erthrocytic protoporphyrin-FEP-or zinc protoporphyrin-ZnP) which is directly proportional to the blood lead levels in patients without iron deficiency (Granick *et al.*, 1972; Chisolm *et*

al., 1974; Piomelli, 1977). Since the erythrocyte has no mitochondria, an increase in FEP reflects bone marrow lead effects and chronic lead poisoning (Sassa et al., 1973b).

ALAD is the other heme biosynthetic enzyme for which there is substantial evidence of lead-induced inhibition. Ever since Bonsignore et al. (1965) unequivocally established that erythrocytic ALAD is markedly inhibited in both acute and chronic lead poisoning, it has repeatedly been demonstrated that the activity of erythrocytic ALAD is inversely proportional to the blood lead level. This in vivo dose-response relationship has been demonstrated in frank as well as in sub-clinical lead poisoning (DeBruin, 1968; Nakao et al., 1968b; Hernberg et al., 1970; Haeger-Aronsen et al., 1971; Weissberg et al., 1971; McIntire et al., 1973) and in the normal urban population as a result of environmental lead pollution (Hernberg and Nikkanen, 1970; Secchi et al., 1971).

The threshold level for the inhibition of ALAD by lead is 15 μg of lead per 100 ml of whole blood (Granick et al., 1973) and this effect is linear to a level of 120 μg of lead per 100 ml of whole blood (Roels et al., 1974). The observation of most investigators that ALAD that is 90% inhibited in vivo or in vitro by relatively high concentrations of lead cannot be further inhibited by lead was confirmed by Goldstein and associates (1975) in baboons administered lead intravenously or orally for nearly six months. The failure

to demonstrate a continued decrease in ALAD activity after 90% inhibition by lead led Weissberg and fellow workers (1971) to conclude that this is the limit of the sensitivity of the assay. However, this may actually represent a natural biochemical process since the -SH groups of ALAD are protected from oxidation in the presence of the substrate ALA (Granick et al., 1973) which may increase in lead poisoning to such a level that it protects that enzyme not yet inhibited by lead. Alternatively, the normal endogenous levels of GSH or some metal activator of ALAD may be high enough to prevent or reverse the lead-induced inhibition of 10% of erythrocytic ALAD.

Aside from providing an extremely sensitive measure of the degree of lead exposure, the inhibition of erythrocytic ALAD is of questionable significance since the erythrocyte lacks the mitochondrial enzymes participating in heme synthesis. However, inhibition of erythrocytic ALAD is probably a reflection of the inhibition of the enzyme in the erythroid precursors of the bone marrow. In addition, a correlation between erythrocytic ALAD activity and the activity of liver ALAD in man (Secchi et al., 1974) and between erythrocytic ALAD activity and the activity of liver and brain ALAD in lead poisoned suckling rats (Millar et al., 1970) has been demonstrated. The consequences of this are as yet undetermined but potentially highly significant.

One known and well established consequence of the

inhibition of ALAD by lead is the marked increase in the urinary excretion of ALA. Urinary ALA excretion rises above normal limits in an exponential fashion in both adults (Haeger-Aronsen, 1960; Selander and Cramer, 1970) and children (Davis et al., 1968). Raised urinary ALA concentrations are closely correlated with plasma levels that are elevated prior to the urinary increase (Haeger-Aronsen, 1960; Chisolm, 1968b). The source of this increased urinary and plasma ALA was suggested by Gibson and Goldberg (1970) who showed that, in rabbits treated with chronic parenteral lead acetate, liver, kidney, and bone marrow ALA increases to levels 2, 12, and 100 times the control levels, respectively The blood lead threshold for the elevation of plasma and urinary ALA is approximately 45 µg of lead per 100 ml of whole blood, which is appreciably higher than that for ALAD inhibition, suggesting the body has a considerable functional ALAD reserve (Lauwerys et al., 1974).

4. THE POSSIBLE CONSEQUENCES OF ALA ACCUMULATION

Acute intermittent porphyria (AIP) is a genetically controlled disease of a chronic nature; the genetic defect involves the overproduction of ALA in the liver as a result of the induction of ALAS (Marver and Schmid, 1972) and a decreased metabolism of ALA to heme due to a partial lack of PD activity (Meyer et al., 1972) resulting in an increased excretion of ALA and PBG. One consequence of the elevated

hepatic ALA biosynthesis and decreased ALA metabolism is an increase in the plasma and cerebrospinal fluid concentrations of ALA (Sweeney et al., 1970). When ALA reaches the serum equilibrium levels seen in AIP it has been demonstrated to pass into various organs of the body including the heart and gut (McGillion et al., 1975). In addition, as suggested by the increase in the cerebrospinal fluid concentration of ALA, the elevated plasma concentration can pass the blood-brain barrier and enter the brain tissue (Kramer et al., 1971; McGillion et al., 1974); Becker et al. (1974), using rabbit brain cortical slices, actually demonstrated brain uptake of ALA via an active transport-like system. Thus in both AIP and lead poisoning there is an increase in tissue and plasma ALA concentrations as well as an increased urinary excretion of ALA.

The signs and symptoms of 50 cases of lead poisoning have been compared with those of 50 cases of patients suffering from AIP and striking similarities have been observed (Dagg et al., 1965). The porphyria triad of abdominal pain, constipation, and vomiting was found to be the most common symptom complex of lead poisoning. The incidence and distribution of non-abdominal pain were nearly identical. In addition, neuropsychiatric manifestations, paresis or paralysis, paresthesias, and cardiovascular abnormalities (including tachycardia and hypertension) were observed in both diseases but the incidence and severity were generally

greater in AIP. Finally, there is a notable similarity of the pathologic changes affecting the nervous system in both diseases which includes a segmental demyelination of the peripheral nerves.

Purified porphyrins and PBG have been demonstrated to be pharmacologically inactive (Goldberg et al., 1954) as has ALA (Jarrett et al., 1956). Nonetheless, the clinical and pathologic similarities between AIP and lead poisoning suggest, despite different etiologies of the diseases, a related pathogenesis. The possibility that the common feature of these diseases, the increased levels of ALA, may be the underlying cause of the clinical and pathologic similarities is, therefore, being reinvestigated. The possible direct toxic effect of ALA in the pathogenesis of an acute attack of AIP (hence, possibly, in lead poisoning) is further implied by the recent observation (Dhar et al., 1975) of a clinical remission and the return to normal of elevated plasma ALA levels after infusion of hematin which, it will be recalled, inhibits ALAS.

In studying the pathogenesis of the hypertension and tachycardia seen in AIP several investigators have found a significant correlation between both blood pressure and pulse rate and the amount of urinary catecholamines excreted (Schley et al., 1970; Atuk et al., 1975). The etiology of the increased excretion of catecholamines was, until recently, unclear. In 1977 Beal and associates demonstrated

a significantly reduced platelet uptake and accumulation of norepinephrine (NE) in the presence of both 1 and 10 μM ALA in patients with AIP but no similar reduction in the platelets of normal patients. The AIP patients' platelets were by no means deficient in uptake capacity since their control uptake of NE was actually greater than that of platelets from normal controls and NE uptake could be significantly decreased in both patients with AIP and normal patients by desmethylimipramine. Both the significantly greater control platelet uptake of NE and the significantly decreased platelet uptake of NE in the presence of ALA led these investigators to conclude that there is a latent defect in catecholamine uptake or accumulation in the platelets of patients with AIP which becomes apparent only in the presence of ALA. Since platelet uptake serves as a model of adrenergic neuronal uptake (Abrams and Solomon, 1969; Born and Smith, 1970) and reuptake is the principle means of terminating the physiologic action of NE *in vivo* (Molinoff and Axelrod, 1971), the elevated circulating catecholamines observed during acute attacks of AIP could be caused, at least in part, by blocked reuptake of catecholamines into adrenergic neurons (Beal *et al.*, 1977). It would be interesting to look for a similar latent defect in those lead intoxicated individuals with hypertension and tachycardia.

Isoniazid produces a pattern of denervation in rats that is similar to that observed in AIP (Cavanagh, 1967).

The principle toxic action of isoniazid is the depletion of pyridoxal phosphate by increasing the excretion of pyridoxal (Holtz and Palm, 1964). It is likely that the neurologic lesion is a result of tissue deprivation of this cofactor since it can be reversed or prevented by pyridoxal phosphate (Zbinden and Studer, 1955). The similar patterns of denervation led Cavanagh and Ridley (1967) to postulate a similar etiology in AIP. That is, the excessive activity of the pyridoxal phosphate-requiring enzyme ALAS leads to a relative tissue depletion of pyridoxal phosphate and the observed neurologic lesion. However, no correlation between plasma pyridoxal phosphate levels and neuropathy was observed in patients with AIP and those with low pyridoxal phosphate levels did not improve after the administration of the vitamin (Hamfelt and Wetterberg, 1968). Thus, while a relative pyridoxal phosphate deficiency might have explained the denervation and, because of decreased levels of gamma-amino-butyric acid (GABA) (due to the inhibition of the pyridoxal phosphate-dependent enzyme of GABA synthesis, glutamic acid decarboxylase) (Roberts, 1963), the other neurologic features of AIP, factors other than this must be responsible.

The structural similarity of ALA and GABA, a ubiquitous inhibitory transmitter in the central nervous system (Johnston, 1978), suggests that ALA might interact with GABA receptors. Nicoll (1976) studied the perfused frog spinal

cord using the sucrose gap technique and found that ALA, added to the preparation in a Ringer solution at pH 7.3, activates GABA receptors on motoneurons, hyperpolarizing them, and on primary afferent fibers, reducing transmitter release from them in response to incoming volleys. The threshold concentration for the effect of ALA reported by Nicoll is 2 to 5 x 10^{-5} M while that for the effect of GABA is 10^{-5} M. Müller and Snyder (1977) found that concentrations of ALA causing postsynaptic and presynaptic inhibition in the frog spinal cord preparation compete with GABA for crude synaptic membrane Na^{+}-independent GABA receptor binding on frog and rat spinal cords as well as on rat brain synaptosomes. Both studies demonstrated the relative potencies of GABA and ALA are more alike in their effect on postsynaptic than on presynaptic inhibition. In addition, Becker et al. (1976) showed that ALA causes an increased release of GABA from both stimulated (at 0.5 μM) and non-stimulated (at 100 μM) rabbit cerebral cortex synaptosomes. Muscimol, a potent GABA-mimicking drug, causes ataxia, muscle weakness, slowing of thought processes, dysphoria, and occasional psychotomimetic effects with hallucinations (Theobald et al., 1968), symptoms reminiscent of the muscle weakness and psychiatric symptoms observed in AIP and lead poisoning (Goldberg, 1959; Roth, 1968). It is therefore possible that some of the symptoms of AIP and lead poisoning may be the result of the effect of ALA on GABA receptors as

well as on GABA release.

A direct presynaptic inhibitory effect of 0.6 to 18 mM ALA at the cholinergic synapse of the rat neuromyal junction was demonstrated in 1979 by Bornstein and fellow workers. Thus, ALA causes a decrease in the number of quanta of acetylcholine (ACh) released by a nerve impulse but has no effect on the depolarization produced by a single quantum of ACh or on the postsynaptic resting membrane potential.

Becker and co-workers (1971) showed that 10^{-4} to 2 x 10^{-3} M ALA inhibits a Na^+, K^+ -activated adenosine triphosphatase (Na,K-ATPase) in rabbit erythrocyte and brain membranes. This enzyme helps to maintain the Na^+ and K^+ gradient across the cell membrane and inhibition by ALA would cause the membrane to become leaky with a resulting decrease in resting membrane potential and a possible block of nerve conduction. More recently Becker and associates (1975), using electrophysiologic techniques, demonstrated that ALA can produce a relatively rapid and large decrease in the resting membrane potential of a single fiber from the sartorius muscle of the frog in a reversible and dose dependent manner over a concentration range of 10^{-4} to 10^{-3} M. This decrease is not likely to be mediated solely by an inhibition of the Na,K-ATPase but probably also involves an alteration in membrane permeability to Na^+ and K^+ by unknown mechanisms possibly involving the release of a depolarizing substance.

The *in vitro* effects described above occur, for the most part, at a concentration (10^{-4} M) at least ten times higher than that typically found in the plasma of patients with lead poisoning and acute attacks of AIP (10^{-6} to 10^{-5} M). This has caused some to question whether circulating ALA is toxic and led to the speculation of another endogenous or induced defect of heme synthesis within the nervous system that is responsible for the symptoms of AIP and lead poisoning (Shanley *et al.*, 1976; Shanley *et al.*, 1977).

D. THE PHYSICAL AND CHEMICAL PROPERTIES OF METAL IONS

1. THE ELECTRON CONFIGURATION (Weidner and Sells, 1968; Cotton and Wilkinson, 1972)

An atom of an element of atomic number Z consists of a small, dense nucleus composed of neutral particles, neutrons, and Z positively charged particles, protons, embedded in a cloud of Z considerably smaller and less massive negatively charged particles, electrons. While the electron cloud is highly dynamic, the arrangement of electrons around the nucleus of an element can be described in terms of occupation of orbitals within successive energy levels. This is referred to as the electron configuration of an element. The physical and chemical properties of an element are largely dependent on its electron configuration and elements with similar electron configurations have similar properties.

Electrons of an atom occupy successive principal energy levels designated by n and of positive integral values with the values of n equal to 1, 2, 3, 4, 5, and 6 corresponding to the K, L, M, N, O, and P shells, respectively. Principal energy levels are divided into sublevels designated by l which has integral values from 0 to $(n-1)$ with the values of l equal to 0, 1, 2, and 3 corresponding to the s, p, d, and f sublevels, respectively. The s, p, d, and f sublevels have $(2l + 1)$ orbitals, each of equal energy but each with a different spatial orientation in the presence

of an external magnetic field. These spatial orientations are designated by the orbital magnetic, m_l, which has the intergral values l, l-1, ..., 0, ..., 1-l, -l. Each orbital can have a maximum of two electrons with parallel and opposite spin magnetics, designated m_s, with a value of either $+\frac{1}{2}$ or $-\frac{1}{2}$.

Although the principal energy levels represent the general order of magnitude of the energy of an electron, the least stable sublevels of one principal energy level may be of higher energy than the most stable sublevels of the next higher level hence will fill after them. Furthermore, according to Hund's Rule orbitals of one sublevel accommodate single electrons of parallel spins in each before the pairing of electrons in the orbitals in that sublevel begins.

The numbers n, l, m_l, and m_s are known as quantum numbers. According to the Pauli Exclusion Principle no two electrons of an atom have the same set of quantum numbers; this is the basis of the structure of the Periodic Table. When the unique set of quantum numbers for each and every electron in an atom is known, the electron configuration of the atom is established. According to the famous Pauli Aufbauprinzip, the different orbitals, each with its unique set of quantum numbers, are arranged in order of increasing energy (1s, 2s, 2p, 3s, 3p, 4s, 3d, 4p, 5s, 4d, 5p, 6s, 4f, 5d, 6p) and the electrons placed into the orbital of lowest

energy until all Z electrons have been accommodated. The periodicity of electron configurations is a result of the recurrent filling of successive principal energy levels; the periodicity of chemical properties reflects the periodicity of the electronic configurations. Each column of the Periodic Table contains elements with similar electron configurations hence similar chemical properties.

Certain electron configurations are more stable than others. The electron configurations of the noble gases, which have two s electrons and six p electrons in their outer shells, are especially stable. Configurations in which subshells are either filled or half filled are also quite stable. It is the tendency of atoms to acquire either closed shell electron configurations of noble gases or filled or half filled subshells through the sharing of electrons with other atoms (covalent bond formation) or through electron transfer (the formation of ionic bonds) that accounts for their oxidation states.

Each electron orbital is a well defined region in space in which the electrons of that orbital are most likely to be found. As mentioned above, each orbital has a different spatial orientation. If one imagines the atomic nucleus to be situated at the origin of three mutually perpendicular Cartesian axes (x, y, and z) it is possible to describe the spatial orientation of the orbitals of the three subshells usually involved in the formation of

chemical bonds. The single s orbital is spherically symmetrical with respect to the origin. The three p orbitals have dumbbell shapes with each orbital oriented along one of the three axes and with the node of the dumbbells at the origin. Four of the d orbitals have cloverleaf shapes; three are oriented in the xy, xz, and yz planes with their lobes situated between the axes and their nodes at the origin while the fourth has its lobes along the x and y axes with its node at the origin. The final d orbital has a dumbbell shape with a torus wrapped around the center; the dumbbell is oriented along the z axis with its node at the origin and the torus is in the xy plane. The set of orbitals of each sublevel are spherically symmetrical with respect to the nucleus. It is the fact that a symmetrical arrangement of electrons about the nucleus is energetically favorable that accounts for the stability of filled or half-filled subshells.

2. THE COORDINATE BOND (Basolo and Johnson, 1964; Basolo and Pearson, 1967)

A coordination compound contains one or more coordinate covalent bonds which are covalent bonds in which one of the two atoms involved supplies both of the electrons of the bond. The electron pair acceptor of the coordinate covalent bond is the central ion and is, typically, an ion of a metal with d orbitals. The coordinating groups, or ligands, are

ions or neutral molecules which are the electron pair donors in the formation of the coordinate covalent bond. The number of coordinate covalent bonds that can exist betwen the central ion and the ligands is the coordination number of the ion.

Alfred Werner laid the foundation of modern structural inorganic chemistry when, in 1893, he proposed his coordination theory. The central principle of this theory, for which Werner won the 1913 Nobel Prize in Chemistry (Werner, 1966), is that most elements have two types of valence, a primary valence corresponding to its oxidation state and a secondary valence corresponding to its coordination number. According to Sidgewick (1927) a central ion, having achieved a stable, non-noble gas electron configuration through oxidation will often surround itself with enough ligands that the total number of electrons around the metal is the same as that in a noble gas. This concept is often useful in predicting the coordination number of a central metal ion.

There are three theories that are used to explain the properties of coordination compounds. These three (the valence bond theory, the electrostatic theory, and the molecular orbital theory) differ in the manner in which they consider the approach of ligands to affect the central atom and its orbitals. However, all three theories qualitatively account for the main features of coordination complexes.

Therefore, the theory that is the most convenient in the present application, the valence bond theory, will be discussed.

Linus Pauling was awarded the 1954 Nobel Prize in Chemistry for developing his theory of chemical bonding (Pauling, 1966). According to his valence bond theory (Pauling, 1960) the number of orbitals on the central ion made available for the formation of coordinate covalent bonds with orbitals on the ligands must equal the number of ligands. Since the strongest coordinate covalent bond is the result of maximum angular overlap of the two coordinating orbitals, the coordinating atomic orbitals of the central metal atom must form hybrid orbitals, all of which are equivalent and have definite spatial arrangements. The geometry of the coordination complexes is established by the geometry of the hybrid orbitals. Thus, for example, if the coordination number is 2, 4, or 6 and the coordinating orbitals are one s and one p, one s and three p's, one d, one s, and two p's, or two d's, one s, and three p's then the respective geometry of the coordinating orbitals and the resultant coordination compounds is linear (sp hybridization), tetrahedral (sp^3 hybridization), square planar (dsp^2 hybridization) or octahedral (d^2sp^3 hybridization).

A σ bond is a coordinate covalent bond formed when a hybrid orbital accepts a pair of electrons from a ligand in such a manner that the electron density is symmetrical about

the bond axis. A π bond, on the other hand, has a minimal electron density about the bond axis and is formed if the central atom has loosely held d electrons and the ligand vacant d orbitals or vice versa. The π bond strengthens the σ bond and reduces the negative charge resulting from the addition of ligands to the central atom.

3. THE HARD AND SOFT ACID AND BASE CLASSIFICATION OF LEWIS ACIDS

G. N. Lewis (1923) defined a base as an atom, molecule, or ion having at least one pair of unshared valence electrons. An acid was similarly defined as an atom, molecule, or ion having a vacant orbital in which a pair of electrons can be accommodated. The typical Lewis acid-base reaction is:

$$A + :B \longrightarrow A:B$$

acid base acid-base complex.

This generalized Lewis acid-base reaction is the most important of all classes of chemical reactions.

The Lewis acid-base concept classifies all metal ions as Lewis acids. Sidgewick (1927) noted that in a classical coordination complex the metal ion completes a stable electron configuration by accepting electron pairs from ligands. He, therefore, named Lewis acid-base reactions involving metal ions with vacant orbitals coordinate bond formation and coined the terms "acceptor" for the Lewis acid or

central metal ion and "donor" for the Lewis base or ligand. It follows that most metal ions tend to form coordination compounds but the tendency to do so generally increases with rising electron affinity of the metal.

Lewis acids and bases have been classified by Pearson (1968 a and b) into hard and soft acids and bases (HSAB) in order to provide a general explanation of the stability of the acid-base complex A:B. The HSAB principle provides a qualitative, or, at best, a semiquantitative rule. It does, nonetheless, have considerable utility, proving very useful in predicting which metal will combine most strongly with which ligand and in correlating and understanding the large amount of information presently available.

Certain properties of the Lewis acid result in either a high electropositivity and low polarizability or low electropositivity and high polarizability. These are general criteria chosen by Pearson as the basis for the classification of Lewis acids as hard or soft. Thus some Lewis acids are small, have a high positive oxidation state, and do not contain unshared electrons in their valence electron shells. These properties result in high electropositivity and low polarizability on the basis of which these Lewis acids are classified as hard. On the other hand some Lewis acids are large, have a low positive oxidation state, and contain several unshared electrons in their valence electron shell.

These properties result in low electropositivity and high polarizability as a result of which these Lewis acids are classified as soft. Borderline acids have intermediate properties hence behave like hard acids in the presence of hard bases and behave like soft acids in the presence of soft bases. Hard solvents, of which water is one of the hardest, will partially destroy hard acid characteristics and enhance soft acid characteristics.

In a similar manner Pearson classified Lewis bases as hard or soft. Hard bases contain a donor atom of low polarizability and high electronegatively which is difficult to reduce and is associated with empty, high energy (hence inaccessible) orbitals. In contrast, soft bases contain a donor atom of high polarizability and low electronegativity which is either easily oxidized or associated with empty, low-lying orbitals.

Pearson formulated the principle of hard and soft acids and bases as a concise statement summing up the experimental data used to classify Lewis acids and bases as hard or soft. That principle states that the most stable complexes result from hard acid-hard base and soft acid-soft base combinations. The type of bond usually associated with hard acid-hard base bond formation is typically ionic while that associated with soft acid-soft base bond formation is typically the covalent (σ) and, under appropriate conditions, the π bond.

"La vie est dure" according to Pearson (1966). That is, hard acids and hard bases are the normal, abundant components of biological systems. Most soft acids and soft bases, on the other hand, are poisons to living organisms because they form strong complexes with the soft bases and soft acids present in small amounts in the organism as the all important active centers of certain enzymes, etc.

E. THE METAL ION AND ITS ROLE IN ENZYME ACTIVITY

1. THE ENZYME (Kachmar and Moss, 1976; Lehninger, 1975)

In 1897 Eduard Buchner discovered that sugar can be made to ferment not only with ordinary yeast but also with the help of the expressed juices of yeast which contain no yeast cells. The discovery of these unknown active substances called enzymes was so important that Buchner was awarded the 1907 Nobel Prize for Chemistry (Buchner, 1966). Wilhelm Ostwald received the 1909 Nobel Prize for Chemistry for formulating the basic principle of catalysis which says that a catalyst increases the rate at which a chemical reaction occurs without itself being consumed or permanently altered (Ostwald, 1966). Ostwald was the first to point out that enzymes act as catalysts. It was not until 1926 when James B. Sumner crystallized the enzyme urease that enzymes were recognized as proteins. For this work Sumner was awarded the 1946 Nobel Prize for Chemistry (Sumner, 1966). It was later demonstrated that the way an enzyme increases the rate of a biological reaction is by providing a reaction route having a lower free energy of activation for the transition of substrate to product than the uncatalyzed reaction.

The specific catalytic activity of many enzymes depends solely on the unique amino acid composition and sequence and the native conformation, or folded state,

determined by the interaction of amino acid side chains with each other and the solvent. Other enzymes, however, require in addition the presence of some nonprotein entities or co-factors before enzyme activity can be manifested or max-imized. If a cofactor is an organic substance it is referred to as a coenzyme while if it is an inorganic ion, such as a metal ion, it is called an activator.

Enzymes are proteins hence heat, extremes of pH, and various chemicals, including metal ions, can alter their structure (i.e. denature them). Denaturation can be wholely or partially reversible or irreversible depending on the cause of the denaturation and its duration of action. Since the ability of an enzyme to catalyze a specific reaction is dependent on its unique amino acid composition and sequence and the precise conformation of the folded polypeptide that they determine, even slight denaturation can render it inactive.

2. THE SULFHYDRYL GROUP AND ENZYME INHIBITION

Sulfhydryl groups (-SH) are chemically the most active groups found in cells. As such they react as a class specifically and often quantitatively with various thiol-combining agents including a wide range of metal ions with which they form stable complexes (Jocelyn, 1972). The reactivity of sulfhydryl groups is due to the fact that they dissociate to mercaptide ions (RS^-) in aqueous media

which are 500 times more nucleophilic that the corresponding oxygen analogue (RO^-) (Streitweiser, 1956). Among factors contributing to the nucleophilicity of the mercaptide group is the ease with which its negative charge and unshared electrons can be polarized and attracted by electrophilic centers. Thus sulfhydryl groups, especially sulfhydryl groups in aqueous media, are soft Lewis bases.

In many enzymes the presence of free cysteine sulfhydryl groups is essential for activity. Boyer (1959) provided a general definition of a sulfhydryl enzyme as an enzyme that loses its catalytic activity when some or all of its original sulfhydryl groups are chemically modified. These, like nearly all sulfhydryl proteins, occur intracellularly and are less stable than disulfide proteins due to the absence of covalent cross links hence they can usually be denatured under mild conditions (Cecil, 1963). In vitro oxidation of these groups to disulfide groups (-S-S-) by dissolved oxygen at neutral and alkaline pH is a typical example of this. Often, however, an enzyme denatured in this manner can be reactivated by treatment with thiols such as cysteine, mercaptoethanol, dithiothreitol, or reduced glutathione which reduce disulfide bridges to the free -SH form through a thiol-disulfide interchange (Haschemeyer and Haschemeyer, 1973).

Sulfhydryl groups within many enzymes vary in reactivity toward various reagents. The reactivity

differences of -SH groups of different enzymes and even within the same enzyme molecule is related to their functional roles as essential catalytic sites in substrate binding and maintaining protein structure and conformation. However, Friedman (1973), after reviewing the literature on more than 40 sulfhydryl enzymes, concluded that the evidence indicating whether the -SH groups of -SH enzymes participate directly in catalysis by, for example, interacting with electrophilic centers of substrates or by maintaining an enzyme conformation required for activity is equivocal. The mechanism is difficult to deduce from current techniques of chemically modifying essential -SH groups because substrate reactions could be sterically hindered by conformational changes near the reactive site as easily as they could by a chemical change in the site itself (Spradlin and Thoma, 1970).

Paul Ehrlich received the 1908 Nobel Prize in Physiology or Medicine for his studies on the relation between toxins and antibodies (Ehrlich, 1967) which resulted in his well-known side-chain theory, the foundation of modern receptor theory. An important conclusion reached by Ehrlich (1909) as a result of this work was that sulfhydryl groups act as receptors for metal ions. Voegtlin and coworkers (1924) later proved this and also demonstrated that thiols could counteract the toxic effect of metal ions by a thiol-mercaptide interchange.

Reactions leading to metal mercaptide formation are

among the most specific of the varied reactions of the sulfhydryl group (Boyer, 1959). In most instances when metal ions are found to inhibit enzymatic activity reaction with sulfhydryl groups is the first suspected. Divalent lead, copper, mercury, cadmium, and zinc and monovalent silver, all soft or borderline Lewis acids, are those metal cations most likely to inhibit enzyme activity through the formation of monodentate metal mercaptides with the sulfhydryl groups of the cysteine residues of a large number of enzymes (Vallee and Wacker, 1970). The marked affinity of certain metal cations for RS^- is a result of their tendency to accept electrons in a coordinate covalent bond together with the willingness of RS^- to donate electrons. In addition, π-type bonding with sulfur can occur through overlap involving d orbitals (Liu, 1977). The relative magnitude of enzyme inhibition due to the interaction of metal ions with free sulfhydryl groups is generally proportional to the solubility products of the metal sulfides (Klotz, 1954).

3. THE METALLOENZYME AND THE METAL-ENZYME COMPLEX

According to Vallee and Wacker (1970) nearly one third of all enzymes have metals built into their structure, require added metal for activity, or are further activated by metal ions. In 1955 Vallee defined metalloenzymes as enzymes which retain stoichiometric, tightly bound, functional metal ions when purified while a metal-activated enzyme was

defined as one to which metal ions had to be added for activity. Malmström and Rosenberg (1959) pointed out the fact that the major difference between metalloenzymes and metal-enzyme complexes is a quantitative one; in metal-activated enzymes the affinity for the metal ion is relatively low but the role of the metal ion might be the same as in metalloenzymes in which the affinity of the enzyme for the metal is relatively high. The rough dividing line between metalloenzymes and metal-activated enzymes is a stability constant of 10^8 M^{-1} (Coleman and Vallee, 1961). Borderline cases do exist in which an enzyme partially retains the metal activator on purification but an increase in the residual activity is observed upon the addition of metal ions to the purified enzyme.

The same metal ion may fill several different roles in the same enzyme and different metal ions may also have different roles in the same enzyme. The structural role, the nature of the ligand, the geometry of the complexes which produce the effects and their relevence to protein conformation are sketchy and cannot be categorized with certainty due to a lack of adequately sensitive experimental approaches.

According to Vallee and Wacker (1970) it is possible for a metal ion to affect enzyme activity by:

a. participating in binding substrates, cofactors, or both

b. activating the enzyme-substrate complex once it is formed

c. serving both functions

d. maintaining secondary, tertiary, or quarternary structure or interacting with the side chains in such a manner that the resulting coordination complex can function catalytically

e. undergoing oxidation-reduction during the enzyme reaction, transferring electrons in oxidative processes

f. bringing about changes in conformation that are either functionally or structurally advantageous or both

g. putting the enzyme in a state of entasis, constituting an energetically poised domain.

For most enzymes the available information does not allow a detailed microscopic differentiation of these possibilities.

In their review of the field Vallee and Coleman (1964) observed that the metals involved in these enzymatic reactions are limited to the alkaline earths, Ca^{+2} and Mg^{+2}, the metals of the second half of the first transition period, the Group IIB metals, and a single metal from the second transition period, molybedenum. There is, according to these authors, no preferred distribution of metals among the various enzyme catalyzed reactions except

for molybdenum and copper. Molybdenum is encountered exclusively in the metalloflavoproteins and copper enzymes catalyze a series of oxidation-reduction reactions without the aid of additional cofactors but copper is not an essential participant in hydrolytic systems. The alkaline earths and manganese activate a large number of enzyme systems involving ATP and in many of these instances the first transition metals can also activate. Zinc plays a varied role in metal-enzyme catalyzed reactions, occurring in each of the six categories of enzymes designated by the International Union on Biochemistry (IUB) commission on enzymes which are oxidoreductases (e.g. human liver alcohol dehydrogenase; Von Wartburg et al., 1964), transferases (e.g. E. coli DNA polymerase; Slater et al., 1971), hydrolases (e.g. human leukocyte alkaline phosphatase; Trubowitz et al., 1961), lyases (e.g. human erythrocyte carbonic anhydrase; Rickli and Edsall, 1962), isomerases (e.g. brewer's yeast mannose-6-phosphate isomerase; Gracy and Noltmann, 1968), and ligases (e.g. yeast pyruvate carboxylase; Scrutton et al., 1970).

The number of side chain functions of proteins (including carboxyl, imidazole, and sulfhydryl functions) , of course, greatly exceeds the number of terminal groups so these are the important metal binding sites. There are always more than are actually needed so each metal ion may choose which site it prefers on a HSAB basis, the general

relationship between protein donor atom and the metal preferring it being:

$-O^-$	$=N$, $-O^-$	$=N$, $-S^-$	$-S^-$
Ca, Mg, Mn	Fe, Co, Ni	Cu, Zn	Cd, Pb, Ag, Hg

The choice of ligand is also determined by factors affecting the local environment of the ligand including intra- or inter-molecular hydrogen bonding, electrostatic or hydrophobic interactions, the vicinal effects of charges, and the stereochemistry of the ligand and the enzyme of which it is a part. Different metal ions can and do combine with the same ligand of a given enzyme but their different geometries could activate the enzyme by providing a three dimensional template or inhibit the enzyme by failing to provide such a template or disrupting the three dimensional structure of the enzyme. (Vallee and Williams, 1968; Williams 1971)

4. THE INTERACTIONS OF METAL IONS IN BIOLOGICAL SYSTEMS

As one considers the trace element requirements of man and animals numerous interrelationships between essential minerals as well as between essential and non-essential minerals are noted. A number of trace element deficiencies arise or are induced by biological interactions among these minerals. A trace element deficiency results from a failure in the supply of an essential nutrient to at least one of its functional sites. When the deficiency is

induced by the presence of antagonists the interaction may occur at any of these essential loci during gastrointestinal absorption and during incorporation into binding sites of enzymes and transport, storage, and cellular structural proteins. Hill and Matrone (1970) have proposed an explanation for these relationships based on the electronic configuration of the ionic form of the minerals. Their hypothesis is that "Those elements whose physical and chemical properties are similar will act antagonistically to each other biologically." The interactions of such elements are, therefore, competitive and, as such, negative and mutual. Thus the effects of an excessive concentration of an antagonistic element, essential or nonessential, is reversible if the concentration of the agonist is increased (Bremner, 1974).

There are antagonistic relationships between the ions of certain elements which have electronic structures that are not at all similar. Such noncompetitive interactions arise when an excess or deficiency of an element influences the metabolic fate of a second element or interferes with some biological process essential for the full expression of its biological activity. This may happen by the excess or deficiency of an element inducing or failing to induce the synthesis of a protein to which the second element binds or by the excess of a given element forming an insoluble, nonavailable complex with the second element. Such

interactions are generally discovered by empirical observation hence there exist no rules concerning reversibility, mutuality, or the negative or positive nature of the interaction. (Davies, 1974)

The present evidence for a direct competition within the gastrointestinal tract for binding sites in the transfer of minerals across intestinal membranes is limited. To be sure, in the presence of abnormally high levels of one element and marginal levels of another indirect competition through the formation of insoluble complexes, a change in solubility as a result of hydrogen ion concentration, or a change in the microflora of the gastrointestinal tract with the consequent sequestering of essential elements can be crucial. Nonetheless, it does seem more likely that absorption competition is minimal when compared with competition at sites within the body tissues (Davis, 1972).

While similar ions are always antagonistic in vivo they often show interchangeability in the activation of certain enzymes in vitro (Matrone, 1970; Vallee and Coleman, 1964). An important area of future research is the identification, in vivo, of synergistic interactions among the elements as well as the substitution of closely related metals in enzyme structures with a consequent change in enzymatic activity.

CHAPTER II

RESEARCH OBJECTIVES

CHAPTER II

RESEARCH OBJECTIVES

The purpose of the research described and discussed in this dissertation was the determination of factors associated with or affecting an increase or decrease in mammalian erythrocytic ALAD activity.

The initial objective of the present research was to determine whether a linear relationship exists between the degree of reticulocytosis and erythrocytic ALAD activity in the developing male rat. This seemed of particular interest because ALAD activity is often used as an index of the degree of experimental lead intoxication in the rat. A variation of ALAD activity with the age of the animal and the reticulocytosis present at that age would be an important source of variability in lead poisoning analysis. Furthermore, if such a relationship does exist this study might serve to establish the developing rat as a useful model in which to further investigate whether a decrease in the activity of the soluble cytoplasmic enzyme with the age of the red cell is due to a denaturation of the enzyme protein, a decreased concentration of an enzyme activator, or an increased concentration of an enzyme inhibitor.

It then seemed necessary to establish a reference value for the erythrocytic ALAD activity of normal, healthy, nonmedicated adult male and female volunteers and to

determine whether the enzyme activity could be correlated with gender, age, or reticulocyte percent. While this would add to the present knowledge that both lead and genetic factors influence the level of erythrocytic ALAD activity, its main purpose was to establish a screen of the enzymatic activity of normal adult human volunteers used in the main section of the present study.

Finally, this research sought to establish a dose-response curve for the in vitro lead-induced inhibition of normal adult human erythrocytic ALAD and for the activation of the enzyme by a metal ion discovered on screening various potential activators. It was, therefore, the main purpose of this study to determine, in a dose-response fashion, the in vitro effects of the soluble acetate, chloride, or fluoride salts of Na^{+}, Mg^{+2}, Al^{+3}, Mn^{+2}, Cu^{+2}, Ag^{+}, Zn^{+2}, Cd^{+2}, Hg^{+2}, Ga^{+3}, In^{+3}, Pb^{+2}, Sn^{+2}, and Sn^{+4} on the normal enzyme and on an enzyme 50% inhibited by Pb^{+2} while at the same time examining the ability of a maximally stimulatory concentration of the activator metal ion to prevent or reverse the effects of these other metal ions. The effects of these metal ions were then classified in terms of their oxidation state, valence electronic configuration, characteristic coordination number, coordination geometry, and hard and soft Lewis acid characteristics in order to determine what physical and chemical properties are associated with the ability of a metal ion to activate or inhibit erythrocytic

ALAD and whether these properties are unique to any single metal ion. This could serve to establish the basis of a future investigation of the actual metal ion requirement of human erythrocytic ALAD as well as of a potential prophylactic and therapeutic use of nontoxic metal salts in the prevention and treatment of both pediatric and adult lead poisoning.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

A. THE WHOLE BLOOD SPECIMEN

1. THE ANIMALS

Male Sprague-Dawley rats were obtained from the Holtzman Rat Company (Madison, Wisc.), rats less than 20 days of age being sent and housed with a nursing dam. Rats more than 20 days old were housed no more than 5 animals per mesh-bottomed stainless steel cage and fed Purina Rat Chow (St. Louis) ad libitum with uninterrupted access to water. Animals were maintained in a temperature controlled (22°C) room under a 12 hour light: 12 hour dark schedule (lights on 0700-1900 hours).

Following ether (Mallinckrodt, St. Louis) anesthesia, blood was obtained from rats aged 20 days and older by aortic puncture using a sodium heparinized Vacutainer tube and an appropriate modification of the technique described below. Blood was obtained from younger rats by decapitation and allowing the blood to drop into a similar Vacutainer tube. A complete blood count and reticulocyte percent were determined for each blood specimen as described below.

2. THE HUMAN VOLUNTEERS

Blood was obtained from normal, healthy, nonmedicated

adult male and female volunteers, after obtaining informed consent, by cubital venipuncture into sodium heparinized Vacutainer tubes as described below. When establishing normal reference values for adult human erythrocytic ALAD activity, volunteers from 18 to 70 years of age were used. For the metal ion activation and inhibition studies, blood was obtained from an equal number of male and female volunteers between the ages of 18 and 30. As a routine screen of the volunteers, a complete blood count and reticulocyte percent were determined for each of the volunteers' blood specimens as described below.

3. THE ROUTINE VENIPUNCTURE (Miale, 1977)

All equipment used for the routine venipuncture was dry and sterile. A tourniquet was applied to the arm of the volunteer above the elbow just tight enough to prevent the venous return. The area over the cubital vein was cleansed by wiping vigorously for one minute with an alcohol wipe (Will Ross, Milwaukee) and allowed to air dry. A 5 ml heparinized (132 units of sodium heparin) B-D Vacutainer tube (3206 KA; Becton, Dickinson and Co., Rutherford, N.J.) was affixed to the adaptor-held 21 gauge/1 inch multidraw needle (Safe II; Jelco, Raritan, N.J.) so the innermost shaft of the needle held the stoppered tube in place but did not penetrate the stopper. The sheath was removed from the needle and the vein entered with the bevel of the needle up

and, with the adaptor held firmly, the Vacutainer tube was forced onto the needle shaft so the rubber stopper was penetrated and the blood flowed into the tube. When the tube was filled to capacity the tourniquet was released, the tube was removed from the needle shaft, and the needle was withdrawn from the vein. The tube was then gently inverted several times to allow thorough mixing of the blood with the anticoagulant, pressure was applied to the site of the venipuncture with a gauze square, and the arm of the volunteer was elevated to assist in collapsing the punctured vein.

B. THE HEMATOLOGIC PROFILE

1. THE MICROHEMATOCRIT

Microhematocrits (Hct) were determined in duplicate using an adaption of the method of Strumia et al. (1954). A plain capillary tube was filled by capillary action approximately two thirds full with an aliquot of heparinized whole blood and one end sealed with sealing clay. The tubes were then placed in opposite radial groves of an International micro-capillary centrifuge, Model MB (International Equipment Company, Needham Hts., Mass.), with the sealed end out against the rubber liner, the centrifuge cover put in place, and centrifuged at 11,500 revolutions per minute. The hematocrit was then read on an International micro-capillary reader. Because the hematocrit measured in this manner includes trapped plasma it is higher than that determined by calculation on the Coulter Counter Model S but the amount of plasma trapping in normal subjects is only 3% (England et al., 1972).

2. THE COMPLETE BLOOD COUNT

The complete blood count (CBC), which includes the hemoglobin (Hb), hematocrit (Hct), red blood cell count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell count, was determined on heparinized whole

blood using the Coulter Counter Model S (Coulter Electronics, Hialeah, Fla.) as described and assessed by Pinkerton and co-workers (1970). This instrument consists, in effect, of three Coulter counters electronically counting and sizing red cells and three electronically counting white cells together with a photosensitive device for measuring Hb concentration as cyanmethemoglobin. From the Hb, red cell count, and MCV, which it measures directly on 44.7 µl of whole blood appropriately diluted by the instrument, the Coulter Counter Model S electronically calculates the Hct, MCH, and MCHC using the following relationships:

$$\text{Hct} = \frac{\text{MCV x red blood cell count}}{10}$$

$$\text{MCH} = \frac{\text{Hb x 10}}{\text{red blood cell count}}$$

$$\text{MCHC} = \frac{\text{Hb x 100}}{\text{Hct}}$$

The precision and accuracy of the results obtained with the Coulter Counter Model S are reported to be much better than that of careful manual estimates and at least comparable to that of other automated equipment.

3. THE RETICULOCYTE COUNT

The new methylene blue method for staining reticulocytes (Brecher, 1949) was employed. The staining solution consisted of 0.5 g of the supravital stain new methylene

blue N and 1.6 g of potassium oxalate (which acts as an anti-coagulant and makes the staining solution isotonic) (Harleco, Gibbstown, N.J.) dissolved in 100.0 ml of distilled water. The solution was filtered before use.

Approximately 100 µl each of staining solution and fresh or heparinized whole blood were mixed in a small cup and allowed to stand for 20 minutes. After staining, thin smears were made and allowed to air dry before counting.

The number of reticulocytes encountered while 1000 erythrocytes were examined under oil immersion was then determined. A reticulocyte was considered to be any erythrocyte containing two or more granular or reticulum-like inclusions with the appropriate staining characteristics since one cannot be certain a single refractile blue granule is a remnant of reticulum (Seip, 1953). The number of reticulocytes counted in this manner divided by 10 was taken to be the percent of reticulocytes present.

C. THE DETERMINATION OF ERYTHROCYTIC ALAD ACTIVITY

1. THE BASIC ALAD ASSAY

All current *in vitro* assays for erythrocytic ALAD are based on the properties of the enzyme described by Gibson *et al*. (1955) and Granick and Mauzerall (1958b). Bonsignore and associates (1965) developed the first clinically useful test for erythrocytic ALAD activity because they demonstrated that it is not necessary to conduct the assay either under nitrogen or *in vacuo* and it is not necessary to wash the red cells. This method was subsequently modified by Weissberg and associates (1971) who conducted the assay on micro samples of blood and by Burch and Siegel (1971) who introduced a more stable buffer. Erythrocytic ALAD was determined in the present study using an adaptation of the whole blood micro-methods of Weissberg *et al*. (1971) and Burch and Siegel (1971) (FIGURE 2).

A 0.1 ml aliquot of heparinized whole blood, drawn into a 100 µl disposable micropipet, was hemolyzed in 1.5 ml of aqeous 0.2% Triton X-100 (octyl phenoxy polyethoxyethanol; Sigma, St. Louis). The hemolysate was warmed to 37°C for 5 minutes following which 1.0 ml of freshly prepared 0.01 M ALA·HCl (Sigma) in 0.25 M dibasic sodium phosphate heptahydrate (Mallinckrodt)/citric acid (Fisher, Fair Lawn, N.J.) buffer. At the end of a 1 hour incubation at 37°C the reaction was stopped by the addition of 1.0 ml of a 10%

trichloroacetic acid (Mallinckrodt) solution which also contained 0.02 M N-ethyl maleimide (Sigma) followed by the immediate addition of 0.05 ml of a saturated cupric sulfate pentahydrate (Baker, Phillipsburg, N.J.) solution. After standing for 5 minutes the mixture was centrifuged at 600 x g for 10 minutes at room temperature. A 2.0 ml aliquot of the supernatant was withdrawn and 2.0 ml of fresh Ehrlich's reagent, prepared as described below, was added. After 15 minutes the optical density was read at 555 nm using a 1.0 cm light path cuvet in a Bausch and Lomb Spectronic 20 colorimeter (Rochester, N.Y.) set to 100% transmission with a reagent blank in which water was substituted for blood since the absorbance of a blank is negligible compared to that of a reagent blank.

A milliunit (mU) of erythrocytic ALAD activity is defined (FIGURE 3) as the amount of enzyme that will catalyze the transformation of 1 nM of ALA to PBG per minute at pH 6.7 and 37°C. Erythrocytic ALAD activity is expressed as mU/ml of packed red blood cells and was defined by the following formula:

$$\text{ALAD mU/ml RBC} = OD_{60} \times (100/\text{Hct}) \times 40.56$$

where:

OD_{60} = the optical density after incubation of the hemolysate with the substrate for 1 hour at 37°C

Hct = the microhematocrit determined as described above

$$40.56 = \frac{(3.65 \times 2)\ (\text{the blood dilution})}{0.1\ (\text{the blood volume}) \times 60\ (\text{the incubation time})}$$

$$\times \frac{1}{0.060\ (\text{the optical density of 1 nM of PBG/ml})}$$

$$\times 2\ (\text{2 molecules of ALA yield 1 molecule of PBG}).$$

The PBG extinction coefficient determined under these experimental conditions is in exact agreement with that described in the literature (Mauzerall and Granick, 1956; Burch and Siegel, 1971).

Triton X-100 is a non-ionic surfactant (soluble amphiphile) that, at the concentration used in the present study, ensures immediate and complete lysis of the erythrocytes. While the molecular mechanism of lysis is unknown, the lytic process is known to consist of five steps:

a. the surfactant adsorbs to the cell membrane
b. the surfactant penetrates into the cell membrane
c. the surfactant induces a change in the molecular organization of the cell membrane
d. the change in molecular organization results in a change in the permeability of the cell membrane and a consequent change in the osmotic equilibrium across the cell membrane
e. once the osmotic equilibrium is sufficiently offset and proteins and other macromolecules begin to pass through the membrane lysis has occurred.

Triton X-100, however, does not induce conformational changes in soluble cytoplasmic proteins leading to a loss of their biological properties hence is safe to use for lysis of erythrocyte membranes so the activity of a soluble cytoplasmic enzyme (such as ALAD) can be studied. (Helenius and Simons, 1975) Mitchell and co-investigators (1977) found that results obtained on erythrocytes hemolyzed in 0.2% Triton X-100 are not significantly different than results obtained on freeze-thaw hemolyzed erythrocytes.

The bicarbonate buffered ALA substrate prepared according to the method of Bonsignore and associates (1965) does not provide adequate buffering capacity, the pH increasing from an initial pH of 7.0 to a final pH of 8.2 after 1 hour of incubation (Burch and Siegel, 1971; Finelli et al., 1974), thus could not be used. In addition, the use of potassium ions in the buffer (as in all other reagents) had to be carefully avoided since K^+ reacts with perchloric acid in the modified Ehrlich's reagent resulting in the formation of a very fine potassium perchlorate precipitate which can interfere with the assay by falsely elevating the absorbance readings (Finelli et al., 1974) unless removed by low-speed centrifugation (Mitchell et al., 1977). Therefore the buffer employed was an extremely stable 0.25 M dibasic sodium phosphate heptahydrate/citrate buffer which maintained the pH of the incubation mixture at the optimum pH found for the normal enzyme, 6.70 ± 0.05 (Burch and Siegel, 1971) even

when very large concentrations of metal ions were added (Mitchell et al., 1977). Furthermore, although phosphates often form insoluble salts with metal cations, the phosphate used in the present buffer system does not interfere with the determination of erythrocytic ALAD activity even when the metal ions are added up to a concentration of 10^{-2} M (Mitchell et al., 1977).

In the determination of erthrocytic ALAD activity the product of the action of ALAD, PBG, is measured colormetrically. In the color reaction PBG condenses at its unsubstituted α position in acidic solution with DMAB, which is present is large excess, to form a colored condensation product, monopyrrylphenylmethane, the intensity of which increases rapidly and diminishes slowly. The color salt can further react with another molecule of PBG to form a colorless dipyrrlphenylmethane but with excess DMAB this reaction is very slow. (Mauzerall and Granick, 1956) Sulfhydryl compounds decolorize the pink salt formed by the reaction of the Ehrlich's reagent with PBG (Granick and Mauzerall, 1958b). Sulfhydryl interference can be prevented by tying up the sulfhydryl groups with N-ethyl maleimide (Burch and Siegel, 1971) or Cu^{+2} (Gibson et al., 1955); in the present procedure both of these methods of removing sulfhydryl interference were employed.

Fresh Ehrlich's reagent was prepared in the following manner (Davis and Andelman, 1967; Davis et al., 1968;

Bio-Rad, 1968): 10 g of p-dimethylaminobenzaldehyde (DMAB; MCB, Norwood, Ohio) was added to 420 ml of glacial acetic acid (Mallinckrodt). This mixture is stable for several months when kept in a refrigerator at 4°C where it will solidify. Subsequent thawing on the day of the analysis was performed by running warm water over the outside of the bottle containing the acetic acid solution containing the added DMAB following which 19 ml of 72% perchloric acid (Mallinckrodt) was added to every 100 ml aliquot of the acetic acid solution containing the added DMAB.

2. THE DETERMINATION OF THE EFFECT OF METAL IONS ON ERYTHROCYTIC ALAD ACTIVITY

The effects of various metal ions, alone and in combinations, on erythrocytic ALAD were studied (FIGURE 4). The metal ions were added by substituting an 0.1 ml aliquot of a freshly prepared solution of a given concentration of the metal ion to be studied for 0.1 ml of the 1.5 ml of aqueous 0.2% Triton X-100 solution in which the whole blood was hemolyzed. Soluble acetate, chloride, or fluoride salts of the metal ions were used after all effects of the acetate, chloride, and fluoride ions on erythrocytic ALAD activity were ruled out. The solutions were prepared in aqueous 0.2% Triton X-100 unless flocculation was observed in which case they were prepared in water. The 0.1 ml aliquots were added immediately after the 5 minute preincubation period or, in

the case of the preincubation studies to be discussed below, before or after the extended preincubation periods. When added in combination an ion being studied for its ability to activate ALAD was always added after an ion known to inhibit ALAD while an ion being studied for its ability to inhibit ALAD was always added before an ion known to activate ALAD.

Several metal ions were initally screened for their effect on human erythrocytic ALAD over a wide concentration range and, as discussed below, Pb^{+2} was identified as an inhibitor of ALAD and Zn^{+2} was identified as an activator of ALAD. The remaining ions were screened, over a broad concentration range, for their effect on the normal enzyme and on an enzyme preparation 50% inhibitied by lead (the ID_{50} Pb^{+2} concentration). In addition, that concentration of Zn^{+2} able to maximally stimulate the enzyme (the SD_{100} Zn^{+2} concentration) was examined for its ability to prevent or reverse the possible inhibition caused by the metal ions being studied.

In order to further investigate the effects of several metal ions of interest preincubation studies were conducted. Solutions of the metal salts were added to hemolysates immediately before or after preincubation periods of 0.5, 1.0, 2.0, and 4.0 hours in order to determine if the observed effect of the metal ion was potentiated, unchanged, or reversed by prolonged exposure of the enzyme to the cation.

Insofar as it was possible, all studies were conducted

in such a manner that paired data could be gathered. That is, for a given blood sample the control ALAD activity was determined for the normal enzyme and, as appropriate, for the enzyme 50% inhibited by Pb^{+2} and maximally stimulated by Zn^{+2} at the same time the effect of metal ions on the normal, Pb^{+2}-inhibited, and Zn^{+2}-stimulated ALAD was being determined on other aliquots of the same blood specimen. All data was expressed as a percent of the normal, uninhibited, unstimulated, nonpreincubated erythrocytic ALAD activity for that specimen but statistical comparisons were made only on raw erythrocytic ALAD activities.

All metal salts used were American Chemical Society approved analytical reagents whenever these were commercially available as were all other reagents. Distilled water was used in the preparation of these and all other aqueous solutions. The metal salts employed were:

$Na(CH_3COO) \cdot 3\ H_2O$ (Mallinckrodt)

NaCl (Baker)

NaF (MCB)

$Mg(CH_3COO)_2 \cdot 4\ H_2O$ (MCB)

$AlCl_3 \cdot 6\ H_2O$ (Mallinckrodt)

$Mn(CH_3COO)_2 \cdot 4\ H_2O$ (MCB)

$Cu(CH_3COO)_2 \cdot H_2O$ (Mallinckrodt)

$Ag(CH_3COO)$ (MCB)

$Zn(CH_3COO)_2 \cdot 2\ H_2O$ (Mallinckrodt)

$Cd(CH_3COO)_2 \cdot 2\ H_2O$ (MCB)

$CdCl_2 \cdot 2\frac{1}{2}\ H_2O$ (Mallinckrodt)

$HgCl_2$ (Mallinckrodt)

$GaCl_3$ (Apache, Rockford, Ill.,)

$InCl_3$ (K & K, Plainview, N.J.)

SnF_2 (Alfa, Danvers, Mass.)

$SnCl_4 \cdot 5\ H_2O$ (Mallinckrodt)

$Pb(CH_3COO)_2 \cdot 3\ H_2O$ (Baker).

D. THE STATISTICAL ANALYSIS

Standard statistical methods were used to determine means, standard deviations, standard errors, nonpaired or Student's t values, paired t values, slopes, intercepts, and correlation coefficients (Goldstein, 1964; Sokol and Rohlf, 1969).

1. THE MEAN, STANDARD DEVIATION, AND STANDARD ERROR

A statistic of location describes the position of a sample along a given dimension representing a variable; such a statistic is, therefore, a measure of central tendency and will yield a representative value for the sample of observations. The arithmetic mean, commonly referred to as the mean or average ($\bar{x}$), is the most important statistic of location. The mean was calculated using the following formula:

$$\bar{x} = \sum_{i=1}^{n} x_i / n$$

where:

n = the total number of observations

$\sum_{i=1}^{n}$ = the sum of the observed values from the first observation through the last.

A statistic of location alone does not completely describe the shape of a frequency distribution; this is done by a statistic of dispersion. The standard deviation, s, is the statistic most commonly employed for describing

dispersion in the same units as those of the mean. The standard deviation is the distance measured along the x-axis from the center of the normal distribution curve (the mean) to the point of inflection of the curve which is its steepest point. The area under the curve at this distance measured on either side of the mean (i.e. $\bar{x} \pm s$) represents 69% of the area of the curve while $\bar{x} \pm 1.96\ s$ represents 95% of the area and $\bar{x} \pm 2.58\ s$ represents 99% of the area. The standard deviation was calculated using the following formula:

$$s = \left(\sum_{i=1}^{n} (x_i - \bar{x})^2 / (n-1)\right)^{\frac{1}{2}}$$

where:

$\sum_{i=1}^{n} (x_i - \bar{x})^2$ = the sum of the squares of the deviations of all of the observed values from the mean of the observations.

The standard error of the mean or the standard error, $s_{\bar{x}}$, is the standard deviation of the sample means and is therefore a measure of the reliability of the estimated mean. It was calculated using this relationship:

$$s_{\bar{x}} = s/(n)^{\frac{1}{2}}.$$

2. THE NON-PAIRED OR STUDENT'S t-TEST

When one wishes to determine whether or not two sample means come from the same population one begins with the null hypothesis and assumes both samples are indeed drawn from

the same population. The hypothesis is rejected if the sample means differ from each other more than random sampling variations would lead one to predict, assuming variances to be homogeneous. To this end the non-paired or Student's t-test was performed which compares the magnitude of the observed difference between sample means with an estimate of its standard error using the formula:

$$t = (\bar{x}_1 - \bar{x}_2)/s_{(\bar{x}_1 - \bar{x}_2)}$$

where:

$\bar{x}_1$ = the mean of sample 1

$\bar{x}_2$ = the mean of sample 2

$s_{(\bar{x}_1 - \bar{x}_2)}$ = the estimate of the standard error of the difference between sample means

$$= (s^2((1/n_1)+(1/n_2)))^{\frac{1}{2}}$$

$$s^2 = (\sum_{i=1}^{n_1}(x_{1i}-\bar{x}_1)^2 + \sum_{j=1}^{n_2}(x_{2j}-\bar{x}_2)^2)/((n_1-1) + (n_2-1))$$

= the pooled variance estimate

n_1 = the total number of observations in sample 1

n_2 = the total number of observations in sample 2

and a table of critical values of t was entered with $((n_1-1) + (n_2-1))$ degrees of freedom for the two-tail test.

3. THE PAIRED t-TEST

When two samples of data are obtained on the same subjects, the variability due to differences between subjects

is eliminated and one considers only the difference in the response of the subjects to a treatment. The paired t-test tests whether the mean of sample differences between pairs of readings is significantly different from a hypothetical mean, which the null hypothesis establishes as zero. The paired t value was determined using the following relationship:

$$t = \bar{d}/s_{\bar{d}}.$$

where:

$\bar{d}$ = the mean difference between the paired observations

$s_{\bar{d}}$ = the standard error of the mean difference, $\bar{d}$

and a table of critical values of t was entered with (n-1) degrees of freedom for a one-tail test since the hypothesis about a treatment effect specifies its direction,

where:

n = the total number of paired observations.

4. THE LINEAR REGRESSION AND CORRELATION COEFFICIENT

The equation of a straight line is:

$$y = mx + b$$

where:

m = the slope of the line or, in a linear regression analysis, the regression coefficient

b = the intercept on the y-axis.

The object of a linear regression analysis is to choose, as a best estimate, a "least squares" line for the data points

by establishing a line from which the sum of the squared deviations of the observed y and the predicted y are minimal. To this end the slope was calculated using this formula:

$$m = \frac{\sum_{i=1}^{n} x_i y_i - \frac{\sum_{i=1}^{n}(x_i) \quad \sum_{i=1}^{n}(y_i)}{n}}{\sum_{i=1}^{n} x^2 - (\sum_{i=1}^{n} x)^2/n}$$

and the y-intercept was calculated using the formula:

$$b = \frac{\sum_{i=1}^{n} y_i - m\sum_{i=1}^{n} x_i}{n} .$$

To determine whether or not a linear correlation between two variables x and y is strong or weak one calculates the correlation coefficient, r. For this purpose the following formula was employed:

$$r = (s_x/s_y)m$$

where:

s_x = the standard deviation of x

s_y = the standard deviation of y.

The value of r may vary from 0, indicating no correlation, to +1 or -1, indicating perfect positive or negative correlation. The significance of r was estimated from the expression:

$$t = (r^2(n-2)/(1-r^2))^{\frac{1}{2}}$$

and a table of critical values of t was entered with (n-2) degrees of freedom for the two-tail test.

5. THE LEVEL OF SIGNIFICANCE

Upon correctly entering a table of critical values of t a P value can be assigned to the difference between sample means, to the difference between the mean of the difference between pairs of observations and the hypothetical difference, and to the strength of a linear correlation which indicates the probability of an observed event occurring by chance. When $P < 0.05$, indicating that the observed event occurs less that 5% of the time by chance, the observation was considered to be significant. When $P < 0.01$, indicating that the observed event occurs less than 1% of the time by chance, the observation was considered to be very significant. When $P < 0.001$, indicating that the observed event occurs less than 0.1% of the time by chance, the observation was considered to be highly significant.

6. THE DISPLAY OF STATISTICAL DATA (Feinstein, 1976)

In order to communicate data what is found should be shown by providing an indication of the central tendency of the data (the mean) and its dispersion (the standard deviation). The standard error is not a summary of evidence hence it is not entirely proper to use it in summarizing data but its use is justified if the reported results are

immediately subjected to inferential testing (tests yielding P values). If no inferential tests are performed the only role of standard errors is to distort and conceal data. This is so because the data looks "tighter" if it is represented as the mean plus or minus the standard error rather than the mean plus or minus the standard deviation which, though larger than the standard error, is truely a summary of the dispersion of the data.

If a graphical illustration of statistical significance is desired a set of 95% confidence interval bars for the means, calculated using the pooled variance estimate for groups as calculated in testing the null hypothesis for the non-paired t-test, is preferred. If the 95% confidence intervals around two means do not overlap the difference between them is significant at $P < 0.05$.

In the present dissertation data is communicated graphically as the mean plus or minus the standard error in order to describe the expected form of the curve not the dispersion of the data. Inferential testing was performed on the raw data from which the graphs were constructed and the level of significance is indicated in the text where it is appropriate. If the reader wishes to know the dispersion of the data about the means in most cases it is only necessary to double the standard error in order to get the standard deviation since the n is usually 4. However, statistically significant differences cannot necessarily be

inferred from the graphs since 95% confidence intervals are not indicated.

CHAPTER IV

RESULTS

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RESULTS

A. THE CORRELATION OF ERYTHROCYTIC ALAD ACTIVITY WITH BLOOD RETICULOCYTE PERCENT IN THE DEVELOPING MALE ALBINO RAT

The variation of several hematologic indices and body weight with the age of the male albino rat is indicated in FIGURE 5. Both the hemoglobin (Hb) and the hematocrit (Hct) were observed to decrease in animals from 3 to 12 days of age. Following the initial drop, both of these values rose steadily from 12 days of age to 50 days of age when these indices were observed to plateau. On the other hand, both the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin (MCH) decreased markedly in animals from 3 to 12 days of age after which the values remained constant for the remainder of the age span studied.

FIGURE 6 illustrates the variation of the blood reticulocyte percent (Rct%) with the age of the male albino rat. As with the MCV and the MCH, a rapid decrease in the reticulocyte percent was observed. The reticulocyte percent decreased from nearly 50% at age 5 days to 35% at 8 days of age and to approximately 25% at 12 days of age. This was followed by a slower decrease to approximately 10% at age 35 days after which the reticulocyte percent appeared to plateau at just under 5%.

The mean peripheral blood values for the developing rat illustrated in these two figures are in excellent agreement with the values reported in the literature (Lucarelli et al., 1964; Lucarelli et al., 1968). The unique picture of a macrocytic hypochromic anemia seen in the early neonatal period of the rat is observed to progress to a normocytic, normochromic state shortly after the onset of puberty at 30 days of age and through the age of 93 days which was the limit of the present study.

Erythrocytic ALAD activity was also found to decrease as a function of the age of the developing male albino rat (FIGURE 7). The ALAD activity of a 5 day old rat was nearly 55 mU/ml RBC. This decreased rapidly to 40 mU/ml RBC at 8 days of age and to 30 mU/ml RBC at 12 days of age. The rapid decrease in erythrocytic ALAD activity during the early neonatal period was followed by a slower decrease to 15 mU/ml RBC at 40 days of age and to approximately 5 mU/ml RBC thereafter up to 93 days of age.

The similarity of the relationship between the reticulocyte percent and the age of the male albino rat to the relationship between the erythrocytic ALAD activity and the age of the male albino rat suggested that there might be a linear relationship between erythrocytic ALAD activity and blood reticulocyte percent in the developing rat. FIGURE 8 shows erythrocytic ALAD activity plotted as a function of the reticulocyte percent for each of the male albino rats

studied. Linear relationship between erythrocytic ALAD activity and the reticulocyte percent was observed which can be described by the equation:

$$y = 1.2x + 3.5$$

where:

y = the erythrocytic ALAD activity in mU/ml RBC

x = the reticulocyte percent.

This linear relationship has a correlation coefficient of 0.967 and a P value of less than 0.001; no significant correlation was observed for the relationship between erythrocytic ALAD activity and any other measurement made in the present study. These data, therefore, indicate that the proportional relationship that exists between erythrocytic ALAD activity and the number of reticulocytes in the peripheral blood of the developing male albino rat is highly significant.

B. THE GENDER-RELATED DIFFERENCES IN THE REFERENCE VALUES FOR THE NORMAL ADULT HUMAN ERYTHROCYTIC ALAD ACTIVITY

The important reference values obtained for the sample population of normal, healthy, nonmedicated adult human male and female volunteers studied, including age, Hb, Hct, Rct%, and erythrocytic ALAD activity are presented in TABLE 1. Blood was obtained from 57 male volunteers having an average age of 36.3 years and from 49 female volunteers having an average age of 37.9 years; there was no statistically significant difference between the mean ages of the male and the female volunteers. There was also no statistically significant difference between the mean reticulocyte percents of the male and the female volunteers, the mean for both genders being 1.2%. There was, however, the expected (Harris and Kellermeyer, 1970) highly significant ($P < 0.001$) difference in the mean Hb and the mean Hct of the male and the female volunteers due to a difference in the level of androgenic hormone (McCullagh and Jones, 1942; Kennedy and Gilbertson, 1957) which enhances the production of erythropoietin as a result of a renotrophic effect (Mann et al., 1968) and enhanced production of renal erythropoietic factor (Rencricca et al., 1969); the mean male Hb of 15.4 g/100 ml and Hct of 45.5% is approximately 10% greater than the mean female Hb of 13.6 g/100 ml and Hct of 40.9%. In contrast, the mean female erythrocytic ALAD activity of 48.2 mU/ml RBC was found to be very significantly ($P < 0.01$) greater than

the mean male erythrocytic ALAD activity of 44.2 mU/ml RBC. These values were normally distributed and there was no statistically significant correlation between erythrocytic ALAD activity and any other measurement made in the present study.

C. THE ACTIVATION AND INHIBITION OF NORMAL ADULT HUMAN ERYTHROCYTIC ALAD BY METAL IONS *IN VITRO*

In light of the numerous contradictory reports concerning the effect of metal ions on ALAD activity it became of interest to investigate the effects of various metal ions on normal adult human erythrocytic ALAD activity *in vitro*. As indicated above, these results were determined only on blood specimens obtained from normal, healthy, nonmedicated adult male and female volunteers with both a normal CBC and a normal erythrocytic ALAD activity.

The first metal ion studied was Cu^{+2} (FIGURE 9), which was found to inhibit ALAD at very low concentrations. A very significant ($P < 0.01$) inhibition of ALAD to 72.5% of the control activity was observed at 9.3×10^{-7} M Cu^{+2} and higher concentrations of Cu^{+2} resulted in highly significant ($P < 0.001$) inhibition of ALAD. That concentration of Cu^{+2} causing 50% inhibition of ALAD (ID_{50}) was determined to be 2.5×10^{-6} M while that concentration of Cu^{+2} causing 90% inhibition of ALAD (ID_{90}) was determined to be 1.5×10^{-5} M.

It seemed quite natural to next determine the effect of Pb^{+2} on erythrocytic ALAD activity (FIGURE 10). Pb^{+2} inhibits ALAD At very low concentrations, a highly significant ($P < 0.001$) inhibition of ALAD was observed at 9.3×10^{-7} M Pb^{+2} and all higher concentrations. The ID_{50} for Pb^{+2} was found to be 1.9×10^{-6} M. While no Pb^{+2} concentration was found to inhibit ALAD more than 90%, the lowest

Pb^{+2} ID_{90} was determined to be 9.3×10^{-6} M.

The effect of Zn^{+2} on human erythrocytic ALAD was determined next (FIGURE 10) and it was found to markedly stimulate ALAD. Over a concentration range of 1.9×10^{-5} M to 9.3×10^{-4} M, Zn^{+2} was found to cause a highly significant ($P < 0.001$) activation of erythrocytic ALAD. The concentration of Zn^{+2} causing a maximum activation of ALAD (SD_{100}) was 3.7×10^{-4} M and at that concentration the activity of ALAD was 157% of the control activity. At concentrations of 1.9×10^{-5} M and above Zn^{+2} inhibited ALAD activity.

Cadmium and mercury are, like lead and copper, heavy metals inhibiting enzymes with functional sulfhydryl groups but they are also, like zinc, members of Group IIB of the Periodic Table hence have physical and chemical properties similar to those of zinc. For this reason it was of interest to discover the effects of Cd^{+2} and Hg^{+2} on ALAD activity (FIGURE 10).

Hg^{+2}, like Pb^{+2} and Cu^{+2}, inhibits ALAD at very low concentrations. At a concentration of 4.0×10^{-6} M, Hg^{+2} caused a very significant ($P < 0.01$) 9% decrease in ALAD activity. At concentrations of 10^{-5} M and above, Hg^{+2} caused a highly significant ($P < 0.001$) decrease in erythrocytic ALAD activity. The ID_{50} for Hg^{+2} was 6.0×10^{-6} M.

Low concentrations of Cd^{+2}, like Zn^{+2}, activate erythrocytic ALAD. At a concentration of 10^{-6} M Cd^{+2} caused a

significant ($P < 0.05$) increase in ALAD activity. Very significant ($P < 0.01$) increases in ALAD activity were observed at the SD_{100}, 4.0×10^{-6} M, and at 10^{-5} M Cd^{+2}, the maximum activity resulting from Cd^{+2} activation being 134% of the control activity. At higher concentrations Cd^{+2} caused a highly significant ($P < 0.001$) inhibition of ALAD, the ID_{50} being 10^{-4} M Cd^{+2}.

Having demonstrated not only the inhibition of erythrocytic ALAD by Pb^{+2}, Cu^{+2}, and Hg^{+2} but also its activation by Zn^{+2} and Cd^{+2}, the obvious question was whether or not an activator of ALAD could prevent or reverse the effect of an inhibitor. To that end the effects of Zn^{+2} on an enzyme inhibited by ID_{50} Pb^{+2} or ID_{90} Pb^{+2} (FIGURE 11) and on an enzyme inhibited by ID_{50} Cu^{+2} or ID_{90} Cu^{+2} (FIGURE 12) were determined and reactivation of the inhibited enzyme was observed.

It was found that Zn^{+2} is able to prevent or reverse the ID_{50} Pb^{+2}- and ID_{50} Cu^{+2}-induced inhibition of erythrocytic ALAD (FIGURE 13). While ID_{50} Pb^{+2} caused a highly significant ($P < 0.001$) decrease in ALAD activity, all concentrations of Zn^{+2} from 1.9×10^{-5} M to 1.9×10^{-3} M caused a highly significant ($P < 0.001$) increase in the activity of the inhibited enzyme. Indeed, at concentrations of 1.9×10^{-4} M Zn^{+2} and above there was no difference from the activity of the Zn^{+2} activated control. In a similar manner, ID_{50} Cu^{+2} caused a highly significant ($P < 0.001$)

decrease in ALAD activity which was prevented or reversed by Zn^{+2} at concentrations from 9.3×10^{-5} M to 9.3×10^{-3} M. While Zn^{+2} caused a highly significant ($P < 0.001$) increase in the activity of ALAD with respect to the activity of the ID_{50} Cu^{+2} control, the reactivation of ALAD was not as complete as that observed for Pb^{+2}-induced inhibition. In fact, the maximum activity observed when Zn^{+2} was added to the ID_{50} Cu^{+2} inhibited enzyme was 106% of the control activity at 3.7×10^{-4} M Zn^{+2}.

The highly significant ($P < 0.001$) inhibition of erythrocytic ALAD by ID_{90} Pb^{+2} was also prevented or reversed by 1.9×10^{-5} M to 1.9×10^{-3} M Zn^{+2} (FIGURE 14). While at Zn^{+2} concentrations below 9.3×10^{-4} M there was a significant decrease from the activities observed in the presence of those concentrations of Zn^{+2} alone, the increases in activity were still highly significant ($P < 0.001$). Indeed, at 1.9×10^{-4} M and 3.7×10^{-4} M Zn^{+2} the ALAD activities were 107% and 134% of the uninhibited control activities, respectively, despite the presence of ID_{90} Pb^{+2}. Zn^{+2} was unable to similarly prevent or reverse the highly significant ($P < 0.001$) ID_{90} Cu^{+2}-induced inhibition. Only at Zn^{+2} concentrations of 3.7×10^{-4} M to 1.9×10^{-3} M were very significant ($P < 0.01$) increases in ALAD activity observed and then the maximum activity observed was only 25% of the uninhibited control ALAD activity at 1.9×10^{-3} M.

These results raised the question of which physical

and chemical properties are associated with the ability of a metal ion to activate or inhibit human erythrocytic ALAD. A natural corollary of this question is whether or not these properties are unique to a single metal ion. In order to answer this question various metal ions having physical and chemical properties similar to those of Zn^{+2} or Pb^{+2} or those previously reported to activate or inhibit ALAD were selected and their effects on both normal human erythrocytic ALAD and on normal erythrocytic ALAD inhibited by ID_{50} Pb^{+2} were determined. In addition, the ability of SD_{100} Zn^{+2} to prevent or reverse the effects of these various ions was determined. In all cases the control inhibition of erythrocytic ALAD by ID_{50} Pb^{+2} and the control activation of the enzyme by SD_{100} Zn^{+2} were at least very significantly ($P < 0.01$) different from the uninhibited and unactivated control erythrocytic ALAD activity.

FIGURE 15 illustrates the results obtained for Pb^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2}. In this figure it will be noted that, while the borderline Lewis acid Pb^{+2} at concentrations of 9.3×10^{-7} M and above cause a highly significant ($P < 0.001$) inhibition of adult human erythrocytic ALAD, 3.7×10^{-4} M Zn^{+2} completely prevented or reversed this inhibition even at very high concentrations of Pb^{+2} where a slight, albeit insignificant, decrease in the activity of the Zn^{+2} activated enzyme was observed. The soft Lewis acid Hg^{+2} similarly caused a highly significant inhibition of ALAD at

concentrations of 4.0×10^{-6} M and greater but this inhibition could not be prevented or reversed by SD_{100} Zn^{+2} and Hg^{+2} did not prevent or reverse the inhibition of ALAD by ID_{50} Pb^{+2}. Instead, the inhibition caused by Hg^{+2} at concentrations above 10^{-5} M was additive with both the stimulation by Zn^{+2} and the inhibition by Pb^{+2} in a highly significant ($P < 0.001$) and a very significant ($P < 0.01$) manner, respectively. As has already been discussed, the borderline Lewis acid Zn^{+2} not only caused a highly significant ($P < 0.001$) activation of ALAD at concentrations from 1.9×10^{-5} M to 9.3×10^{-4} M but also completely prevented or reversed the effects of ID_{50} Pb^{+2} over a concentration range of 1.9×10^{-5} M to 1.9×10^{-3} M. The soft Lewis acid Cd^{+2} caused a significant ($P < 0.05$) to highly significant ($P < 0.01$) increase in ALAD activity over the concentration range of 10^{-6} M to 10^{-5} M. Cd^{+2} from 10^{-5} M to 4.0×10^{-4} M also prevented or reversed the ID_{50} Pb^{+2}-induced inhibition of ALAD, causing a very significant ($P < 0.01$) increase in ALAD activity to as high as 111% of the uninhibited control activity at 10^{-5} M. At concentrations of 4.0×10^{-5} M and above Cd^{+2} caused a highly significant ($P < 0.001$) inhibition of ALAD that could not be prevented or reversed by SD_{100} Zn^{+2}.

In FIGURE 16 the effects of the hard Lewis acids Na^{+}, Mg^{+2}, Al^{+3}, and Mn^{+2} on normal, ID_{50} Pb^{+2} inhibited, and SD_{100} Zn^{+2} stimulated ALAD are illustrated. It will be

noted that over a concentration range of 1.9×10^{-6} M to 1.9×10^{-3} M Na^{+} had no effect on the normal, Pb^{+2} inhibited, or Zn^{+2} activated enzyme. Similarly, Mg^{+2} and Mn^{+2} had no effect on the normal, Pb^{+2} inhibited, or Zn^{+2} activated enzyme at concentrations from 10^{-7} M to 10^{-3} M. Except at concentrations above 4.0×10^{-3} M Al^{+3} had no effect on normal, Pb^{+2} inhibited, or Zn^{+2} activated ALAD. At 10^{-2} M Al^{+3} caused a highly significant ($P < 0.001$) decrease in ALAD activity to 49% of the control activity hence this was considered to be the Al^{+3} ID_{50}. This inhibition by Al^{+3} could not be prevented or reversed by SD_{100} Zn^{+2}, rather 4.0×10^{-3} M and 10^{-2} M Al^{+3} caused a highly significant ($P < 0.001$) decrease in the activity of the Zn^{+2} activated enzyme. Similarly, the Al^{+3}-induced inhibition of ALAD was additive with that produced by ID_{50} Pb^{+2} and no concentration of Al^{+3} prevented or reversed the ID_{50} Pb^{+2}-induced inhibition of ALAD.

Monovalent silver is a soft Lewis acid with the same d^{10} valence electronic configuration found in Zn^{+2}, Cd^{+2}, and Hg^{+2}. However, Ag^{+} (FIGURE 17), unlike Zn^{+2} and Cd^{+2} but like Hg^{+2}, was only found to inhibit ALAD in the concentration range studied. At concentrations of 2.0×10^{-6} M and greater Ag^{+} caused a highly significant ($P < 0.001$) decrease in ALAD activity, the ID_{50} being 2.0×10^{-6} M. Ag^{+} did not prevent or reverse the inhibition of ID_{50} Pb^{+2} nor could the Ag^{+}-induced inhibition by prevented or reversed by

SD_{100} Zn^{+2}. Rather, the Ag^{+}-induced inhibition was additive with the activation by Zn^{+2} and the inhibition by Pb^{+2} in a highly significant ($P < 0.001$) manner.

Trivalent gallium and indium also have $\underline{d}^{10}$ valence electronic configurations but, unlike the other $\underline{d}^{10}$ ions already discussed which are soft or borderline Lewis acids, Ga^{+3} and In^{+3} are hard Lewis acids. Both Ga^{+3} and In^{+3} (FIGURE 18) caused a very significant ($P < 0.01$) inhibition of adult human erythrocytic ALAD activity at concentrations of 4.0×10^{-4} M and 10^{-3} M, the ID_{50} for both being 7.0×10^{-4} M. The inhibition produced by both Ga^{+3} and In^{+3} could be prevented or reversed by SD_{100} Zn^{+2} throughout the concentration range studied. Ga^{+3} and In^{+3} were, however, unable to prevent or reverse the inhibition of ALAD by ID_{50} Pb^{+2}, the inhibition by both Ga^{+3} and In^{+3} actually caused a very significant ($P < 0.01$) increase in the Pb^{+2}-induced inhibition of erythrocytic ALAD.

The effects of the last metal ions studied, Sn^{+2} and Sn^{+4}, are illustrated in FIGURE 19. The borderline Lewis acid Sn^{+2} has the $\underline{s}^2\underline{d}^{10}$ valence electronic configuration of Pb^{+2} and, like Pb^{+2}, caused an inhibition of erythrocytic ALAD at very low concentrations that could be prevented or reversed by Zn^{+2}. At concentrations of 10^{-5} M and above Sn^{+2} caused a highly significant ($P < 0.001$) inhibition of ALAD, the ID_{50} for Sn^{+2} being 4.0×10^{-5} M. The Sn^{+2}-induced inhibition of ALAD could be completely prevented or

reversed by SD_{100} Zn^{+2} up to 10^{-3} M Sn^{+2} where a very significant ($P < 0.01$) decrease in the activity of the Zn^{+2} activated enzyme was observed. Sn^{+2} could not prevent or reverse the ID_{50} Pb^{+2}-induced inhibition but the inhibition produced by Sn^{+2} at concentrations of 4.0×10^{-5} M and greater was additive with that produced by ID_{50} Pb^{+2}. The hard Lewis acid Sn^{+4} has the same valence electronic configuration as Ag^{+}, Zn^{+2}, Cd^{+2}, Hg^{+2}, Ga^{+3} and In^{+3}. Sn^{+4} caused a very significant ($P < 0.01$) inhibition of ALAD only at a concentration of 4.0×10^{-4} M and above and this inhibition was additive with the SD_{100} Zn^{+2} stimulation. At a concentration of 10^{-4} M Sn^{+4} was able to cause a highly significant ($P < 0.001$) reversal of the ID_{50} Pb^{+2}-induced inhibition but only to 67% of the uninhibited control ALAD activity. At 10^{-3} M the inhibition produced by Sn^{+2} caused a very significant ($P < 0.01$) increase in the inhibition caused by ID_{50} Pb^{+2}.

D. THE EFFECT OF PREINCUBATION ON THE ACTIVATION AND INHIBITION OF NORMAL ADULT HUMAN ERYTHROCYTIC ALAD BY METAL IONS IN VITRO

In order to further investigate the interesting effects of Pb^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2} on normal human erythrocytic ALAD, various concentrations of these cations were added to hemolysates which were then incubated at 37°C for 1.0 hour before adding the substrate. These results are compared with the effect of these metal ions on normal adult human erythrocytic ALAD activity when they were added to hemolysates immediately prior to adding the substrate. The activity of control hemolysates incubated for 1.0 hour at 37°C before adding the substrate was not significantly different than the activity of a nonpreincubated control hemolysate.

FIGURE 20 illustrates the effect of various concentrations of Pb^{+2} and Hg^{+2} on normal adult human erythrocytic ALAD when added without preincubation and before a 1.0 hour preincubation of the hemolysate. As indicated in this figure, a 1.0 hour preincubation enhanced the inhibition of erythrocytic ALAD by Pb^{+2}, causing a very significant ($P < 0.01$) decrease in the ID_{50} from 1.9×10^{-6} M Pb^{+2} without preincubation to 6.2×10^{-7} M with a 1.0 hour preincubation. A 1.0 hour preincubation of the whole blood hemolysate with Hg^{+2} similarly enhanced the ability of Hg^{+2} to inhibit ALAD, causing a very significant ($P < 0.01$) decrease in the ID_{50}

for Hg^{+2} from 6.0×10^{-6} M without preincubation to 1.9×10^{-6} M with a 1.0 hour preincubation.

FIGURE 21 illustrates the effects of a 1.0 hour preincubation with the metal ion on the activation of erythrocytic ALAD by Zn^{+2} and on the activation and inhibition by Cd^{+2}. A 1.0 hour preincubation with the hemolysate had no significant effect whatsoever on the ability of Zn^{+2} to activate erythrocytic ALAD. On the other hand, a 1.0 hour preincubation of the enzyme with Cd^{+2} not only caused a very significant ($P < 0.01$) enhancement of the Cd^{+2}-induced inhibition of ALAD, changing the ID_{50} from a non-preincubation concentration of 8.0×10^{-5} M to 2.5×10^{-6} M with a 1.0 hour preincubation, but it also completely prevented the Cd^{+2} activation of erythrocytic ALAD. The Cd^{+2} SD_{100} caused a very significant ($P < 0.01$) activation of erythrocytic ALAD to 124% of the control activity when added without preincubation but caused a highly significant ($P < 0.001$) decrease to 42% of the control activity when preincubated with the enzyme for 1.0 hour.

Additional experiments were conducted in which various concentrations of Pb^{+2}, Cd^{+2}, or Zn^{+2} were added to the hemolysates without preincubation or prior to preincubation for 0.5, 1.0, 2.0, and 4.0 hours at 37°C before adding the substrate. The activities of control hemolysates preincubated for 0.5 or 1.0 hour prior to adding the substrate were not significantly less than the activity of a nonpreincubated

control hemolysate but the activities of control hemolysates preincubated for 2.0 or 4.0 hours were.

The enhancement of Pb^{+2}-induced inhibition previously described for a 1.0 hour preincubation with Pb^{+2} is already apparent after a 0.5 hour preincubation with Pb^{+2} and becomes more marked with 2.0 and 4.0 hours of preincubation (FIGURE 22). That this is not due solely to denaturation of the enzyme on preincubation is suggested by FIGURE 23 in which the results of the preceeding figure are plotted as a function of the preincubation time. While the control activity decreases after 2.0 and 4.0 hours of preincubation, the inhibition induced by various concentrations of Pb^{+2} increases at a faster rate beginning at earlier preincubation times.

In a similar manner, the prevention of the activation of ALAD by Cd^{+2} and the enhancement of Cd^{+2}-induced inhibition as a result of a 1.0 hour preincubation of the hemolysate with Cd^{+2} is notable after 0.5 hour of preincubation with the enzyme and is more marked after 2.0 and 4.0 hours of preincubation (FIGURE 24). Again, when these results are represented as a function of the preincubation time (FIGURE 25), it can be seen that this is apparently a true preincubation phenomenon and not solely due to denaturation of the enzyme as a result of the preincubation.

As can be seen in FIGURES 26 and 27, preincubation of the hemolysate with Zn^{+2} for 0.5, 1.0, 2.0, and 4.0 hours

had little effect on the Zn^{+2} activation of human erythrocytic ALAD. This is in marked contrast to the effects of preincubation on the effects of Pb^{+2} and Cd^{+2} on ALAD.

Further experiments were conducted to determine whether the enhancement of Pb^{+2}-, Hg^{+2}-, and Cd^{+2}-induced inhibition and the prevention Cd^{+2} activation of ALAD after preincubation with these cations is the result of denaturation of the enzyme during preincubation, rendering it more susceptible to inhibition, or a true preincubation phenomenon. In these experiments the effect of various concentrations of Pb^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2} on ALAD activity was determined when these ions were added without preincubation, before a 1.0 hour preincubation, and after a 1.0 hour preincubation (FIGURE 28). It was found that there is little, if any, difference between the results obtained when the metal ions were added without a 1.0 hour preincubation or after a 1.0 hour preincubation. Similarly, the results obtained when metal ions were added after a 1.0 hour preincubation were as significantly different from those obtained when the metal ions were added prior to a 1.0 hour preincubation as the results obtained when the metal ions were added without preincubation were. It, therefore, seems apparent that the enhancement of the Pb^{+2}-, Hg^{+2}-, and Cd^{+2}-induced inhibition and, especially, the elimination of the Cd^{+2} activation may indeed be the result of a true preincubation phenomenon and not of protein denaturation.

The final consideration of the present research was to determine whether or not preincubation would interfere with the ability of Zn^{+2} to affect the reactivation of Pb^{+2} inhibited ALAD. The ID_{50} of Pb^{+2} determined under nonpreincubation conditions was added to the hemolysates after preincubation for 1.0 hour with various concentrations of Zn^{+2}. In addition, various concentrations of Zn^{+2} were added to hemolysates after preincubation for 1.0 hour with the preincubation ID_{50} Pb^{+2}. Finally, the preincubation ID_{50} Pb^{+2} and various concentrations of Zn^{+2} were added to the hemolysate prior to preincubation for 1.0 hour.

As can be seen in FIGURE 29, Zn^{+2} is able to completely prevent or reverse the effect of a preincubation ID_{50} Pb^{+2} whether Zn^{+2} was added after a 1.0 hour preincubation with Pb^{+2} or was preincubated simultaneously with Pb^{+2}. A Zn^{+2} concentration of 3.7×10^{-4} M reversed the Pb^{+2}-induced inhibition of ALAD to 132% of the uninhibited, nonpreincubated control activity when added after preincubation with Pb^{+2} and to 137% of the control activity when preincubated simultaneously with Pb^{+2}.

It will be noted in FIGURE 30 that preincubation with Zn^{+2} was able to completely prevent the inhibitory effects of Pb^{+2} on ALAD whether the inhibition was caused by preincubation ID_{50} Pb^{+2} or by nonpreincubation ID_{50} Pb^{+2}. A concentration of 3.7×10^{-4} M Zn^{+2} reversed the Pb^{+2}-induced inhibition of ALAD to 140% of the uninhibited, nonpreincubated

control activity when Pb^{+2} was added after preincubation with Zn^{+2} and to 137% of the control activity when preincubated simultaneously with Pb^{+2}.

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

A. THE IMPLICATIONS OF THE ASSOCIATION OF THE DECREASE IN ERYTHROCYTIC ALAD ACTIVITY WITH THE DECREASE IN RETICULOCYTE PERCENT IN THE DEVELOPING MALE ALBINO RAT

Because a decrease in erthrocytic ALAD activity is often used as an index of the degree of lead intoxication, the correlation of a decrease in the activity of erythrocytic ALAD with a decrease in the reticulocyte percent as the normal male albino rat develops could be an important source of variability in the analysis of the lead intoxication of newborn and developing rats. This should, therefore, be carefully controlled for or the erythrocytic ALAD assay should be completely avoided when evaluating the degree of experimental lead intoxication of newborn and developing male (and, presumably, female) albino rats.

The prolonged elevation of erythrocytic ALAD activity and reticulocyte percent observed in the developing rat may well reflect a prolonged net peripheral heme biosynthesis; whether or not it actually does remains to be investigated. In light of this possibility, a consideration of the age of the animal might also be in order when using the newborn and developing male albino rat as a model for other disorders of heme synthesis including the porphyrias.

The association of a decrease in erythrocytic ALAD activity with a decrease in the reticulocyte percent as the male albino rat develops suggests that with the maturation of the erythrocyte there is a gradual decline in the activity of the soluble cytoplasmic enzyme ALAD in addition to the expected loss of the mitochondrial heme biosynthetic enzymes. While the association of a high ALAD activity with a large population of young cells capable of synthesizing protein suggests the loss of ALAD activity as the nonnucleated cell population ages is due to protein degradation or to protein denaturation (due to such factors as oxidation of -SH groups to -S-S- bonds or to dissociation of the enzyme subunits) it certainly does not prove it. The decline in erythrocytic ALAD activity with a decrease in the reticulocyte percent and an increase in the age of the animal may reflect an increased body burden of the ubiquitous environmental contaminant and ALAD inhibitor Pb^{+2} as the animal ages. The decrease in the ALAD activity with a decrease in the reticulocyte percent and increase in the age of the animal may also reflect a difference in the content or metabolism of a cofactor of ALAD (such as Zn^{+2}-see below) in the young versus the old cells or to a difference in the serum levels or whole animal metabolism of such a cofactor at various stages of development. Such possibilities could be explored in this animal model and could possibly provide some insights into one of the basic processes of ageing-the

alteration of enzymes (Rothstein, 1975). This model could be especially useful for the study of the alteration of enzymes on ageing since the role of sequence changes during protein synthesis in this process could be more easily controlled for than in other model systems in which the cells are nucleated.

Abdulla et al. (1978a) have recently confirmed the observation of a very high erythrocytic ALAD activity in rats after partus and of a marked decrease in the activity of the enzyme during the early growth period of the rat. While these investigators could not measure plasma and whole blood Zn^{+2} concentrations in young animals, they did find that animals less than six weeks old when fed a zinc deficient diet had a significantly lower ALAD activity than those animals fed a diet containing adequate levels of zinc. In older animals, on the other hand, the level of dietary zinc had no effect on erythrocytic ALAD activity. In addition, since hepatic zinc plays an active role in zinc homeostasis (Abdulla et al., 1978b) and hemoglobin synthesis during fetal and early developmental growth in the rat occurs in the liver, these investigators examined the zinc status of the liver and found that the livers of newborn rats had zinc levels three times higher than those of adult rats. This, together with the finding that in zinc deficiency the hepatic zinc levels were also decreased, may offer some explanation of the differences in ALAD activity

as the animal develops. More work certainly remains to be done to further investigate this.

In the same article Abdulla and associates also reported the observation that the erythrocytic ALAD activity of human infants is nearly one and one-half times that of the adult human. Plasma Zn^{+2} concentrations of the newborn are 25% higher than those of the adult while whole blood Zn^{+2} levels are 50% lower than those of the adult. This is consistent with the earlier observation (Haeger-Aronsen et al., 1976) that erythrocytic ALAD activity could be strongly correlated with plasma Zn^{+2} levels and more weakly correlated with erythrocyte Zn^{+2} levels. However, this also raises an interesting question needing further clarification because the plasma Zn^{+2} levels reported by these investigators for adult rats is significantly higher than that reported for the adult human yet the adult rat has a much lower erythrocytic ALAD activity than has the adult human.

B. THE POSSIBILE EXPLANATIONS FOR THE GENDER-RELATED DIFFERENCE IN NORMAL ADULT HUMAN ERYTHROCYTIC ALAD ACTIVITY

The biological significance of the gender-related difference in normal adult human erythrocytic ALAD activity is minimal on the basis of the present information since it has been suggested that there is excess enzyme in the body (Lauwerys et al., 1974) and, more importantly, because the mature erythrocyte does not synthesize heme. However, this sex-related difference could reflect higher absolute levels of the enzyme in the erythroid precursors and the other cells of the body, possibly as a reflection of a higher rate of porphyrin synthesis in the female. The gender-related difference in erythrocytic ALAD activity could also be a reflection of higher plasma or whole blood concentrations or differences in the metabolism of a cofactor of ALAD (such as Zn^{+2} or Cu^{+}-see below) or a lower concentration of an inhibitor of ALAD (such as Pb^{+2}).

The possibility that the sex-related difference in erythrocytic ALAD activity is due to a sex-related difference in the blood lead level is unlikely since it has been found, after an exhaustive international study, that the blood level of normal adult males is not significantly different than that of the normal adult females (Goldwater and Hoover, 1967). Similarly, the possibility that this difference is due to a sex-related difference in the levels of zinc, a possible activator of ALAD, is unlikely since it

has been reported that there are no gender-related differences in whole blood, erythrocytic, or plasma zinc levels (Vallee and Gibson, 1948; Halsted and Smith, 1970). A possible explanation of the sex-related difference in erythrocytic ALAD activity that merits further consideration is the fact that female serum copper levels are significantly higher than male serum copper levels (Lahey et al., 1953; Bloomfield and MacMahon, 1969); this relationship is particularly interesting in light of the possibility, discussed below, that Cu^{+} is the true in vivo activator of ALAD.

Until further research explains the reason for the difference between the mean male and female erythrocytic ALAD activities, this sex-related difference should be considered when establishing reference values for the erythrocytic ALAD assay used as a diagnostic aid in the evaluation of lead intoxication.

C. THE CONSIDERATIONS REGARDING THE EFFECT OF METAL IONS ON NORMAL ADULT HUMAN ERYTHROCYTIC ALAD ACTIVITY IN VITRO

1. THE GENERAL CONCLUSIONS CONCERNING THE PHYSICAL AND CHEMICAL PROPERTIES ASSOCIATED WITH THE ABILITY OF METAL IONS TO ACTIVATE OR INHIBIT HUMAN ERYTHROCYTIC ALAD IN VITRO

The findings of the present study that low concentrations of Cu^{+2}, Ag^{+}, Hg^{+2}, Sn^{+2}, and Pb^{+2} inhibit normal adult human erythrocytic ALAD in vitro at very low concentrations is entirely consistent with the reports of others (Calissano et al., 1965; Tomio et al., 1968; Chiba and Kikuchi, 1978) and is not surprising in light of the sulfhydryl nature of ALAD. Zn^{+2} activation of human erythrocytic ALAD is also consistent with the reports of others of the Zn^{+2} activation of ALAD from human and other mammalian sources (Abdulla and Haeger-Aronsen, 1971; Cheh and Neilands, 1973; Finelli et al., 1974) as is the prevention or reversal of Pb^{+2}-induced inhibition of ALAD by Zn^{+2} (Finelli et al., 1975). Indeed, recent reports have confirmed the observation of Zn^{+2} activation of ALAD (Mitchell et al., 1977) as well as the protective effect of Zn^{+2} against Pb^{+2}-induced inhibition of ALAD (Border et al., 1976b, Haeger-Aronsen, et al., 1976; Cantrell et al., 1977).

Normal human whole blood concentrations of zinc are $1.35 \pm 0.31 \times 10^{-4}$ M (Vallee and Gibson, 1948) which corresponds favorably with the in vitro concentrations of

zinc used in the present study. Normal whole blood concentrations of lead are 0.7-1.9 x 10^{-6} M (Goldwater and Hoover, 1967) which are also within the range of the *in vitro* concentrations of lead used in the present study; indeed, lead has been reported to inhibit human erythrocytic ALAD *in vivo* at whole blood concentrations much lower than the supposedly safe upper limit of normal (Hernberg *et al.*, 1970). In addition, evidence of clinical lead toxicity can be observed at whole blood lead concentrations above 2.8 x 10^{-6} M (Gibson *et al.*, 1968).

The suggestion that the *in vitro* addition of Cd^{+2} can stimulate ALAD activity (Wilson *et al.*, 1972; Cheh and Neilands, 1973; Hamp and Kriebitzsch, 1975) was unequivocally demonstrated in the present study and was later confirmed (Mitchell *et al.*, 1977). That Cd^{+2} is able to prevent or reverse Pb^{+2}-induced inhibition of ALAD is a unique finding of the present investigation. Preincubation studies in the present work, however, give reason to doubt the possibility that activation of ALAD by Cd^{+2} might be observed *in vivo* since prolonged exposure of the enzyme to Cd^{+2} causes enzyme inhibition.

While the activation of ALAD by 0.18 mM Zn^{+2} reported by Meredith and associates (1974) was exactly duplicated in this study their often cited observation of Al^{+3} activation of ALAD was not. This discrepancy might be explained by their use of a potassium phosphate buffer in their assay and

is not unexpected in light of the hard Lewis acid character of Al^{+3} and the presence of many soft Lewis base sites, the sulfhydryl groups, on the enzyme.

The reported activation of human erythrocytic ALAD by Mn^{+2} (Collier, 1971) could not be reproduced in the present investigation. That the hard Lewis acid Mn^{+2} has no effect on ALAD except at very high concentrations was recently confirmed (Mitchell et al., 1977).

The report by Mitchell et al. (1977) of the activation of ALAD by 10^{-5} M Hg^{+2} is inconsistent with the already mentioned reports of others of inhibition of ALAD by Hg^{+2} at these concentrations and could not be reproduced in the present study. The findings of Mitchell and co-workers is contrary to what is expected on the basis of the properties of Hg^{+2} for, while Hg^{+2} has the same valence electronic configuration as Cd^{+2} and Zn^{+2}, in vitro activators or erythrocytic ALAD, its chemical properties are quite different than other members of Group IIB of the Periodic Table, as discussed below.

The metal ions Na^{+}, Mg^{+2}, Ga^{+3}, In^{+3}, and Sn^{+4} were not found to have any significant effect on human erythrocytic ALAD activity except at very high concentrations and there is no report in the present literature of any significant effect of these metal ions on the ALAD from any other mammalian source.

TABLE 2 lists the physical and chemical properties of

metal ions generally considered of importance in the interaction of metal ions with proteins for each of the 14 metal ions investigated in the present study. These properties include oxidation state, valence electronic configuration, characteristic coordination number, coordination geometry, and Pearson HSAB classification of Lewis acids. In addition, TABLE 2 summarizes the effects of these metal ions on erythrocytic ALAD by listing the ID_{50} and the SD_{100}. Several important conclusions concerning the physical and chemical properties associated with the ability of a metal ion to activate or inhibit erythrocytic ALAD in vitro can be reached on the basis of the above results.

On the basis of the present study it can be concluded that hard acids have no effect on erythrocytic ALAD in vitro except at concentrations approaching the millimolar range, very high concentration for a metal-protein interaction. Thus even those hard acids with the same valence electronic configurations as Zn^{+2} and Cd^{+2} (i.e. Ga^{+3}, In^{+3}, and Sn^{+4}) or with the same oxidation state as Zn^{+2} and Cd^{+2} (i.e. Mg^{+2} and Mn^{+2}) were found to be without effect on erythrocytic ALAD except at very high concentrations. This implies that activation and inhibition of ALAD is due to the interaction of the metal ions with a soft Lewis base of the enzyme, such as the sulfhydryl group, rather than with a hard Lewis base.

The present evidence indicates that borderline or soft Lewis acids having a planar coordination geometry and

either a characteristic coordination number that does not give the ion the electronic configuration of a noble gas, hence have empty p orbitals into which electrons can be donated by a ligand in a π bond, or a characteristic coordination number that gives the ion the electronic configuration of a noble gas and a geometry that permits donation of d electrons to a ligand in a π bond which reduces the negative charge on the central ion, inhibit ALAD at very low concentrations. Thus, despite having the same valence electronic configuration as Zn^{+2} and Cd^{+2}, the soft Lewis acids Ag^{+} and Hg^{+2}, with a characteristic coordination number of 2 (which will not give them the electronic configuration of a noble gas that a coordination number of 4 would) and a linear coordination geometry, inhibit erythrocytic ALAD. Similarly, the borderline Lewis acids Sn^{+2} and Pb^{+2}, despite having the same oxidation state as Zn^{+2} and Cd^{+2}, have a planar coordination geometry (Ψ-tetrahedral) which permits the donation of d electrons to ligands in a π coordinate bond and inhibit the enzyme. These Lewis acids could, therefore, form not only a σ coordinate-covalent bond with a soft Lewis base of the enzyme, such as the sulfhydryl group, but also a much more stable bond by π bond formation through either accepting electrons from sulfur into empty p orbitals (i.e. Ag^{+} and Hg^{+2}) or donating electrons into an empty d orbital of sulfur (i.e. Sn^{+2} and Pb^{+2}), thereby inhibiting the enzyme.

Of the 14 metal ions studied, only the borderline Lewis acid Zn^{+2} and the soft Lewis acid Cd^{+2} activate erythrocytic ALAD *in vitro* and prevent or reverse the *in vitro* Pb^{+2}-induced inhibition of erythrocytic ALAD. These are divalent, d^{10} Lewis acids with a characteristic coordination number of 4 that gives them the electronic configuration of a noble gas and a nonplanar coordination geometry (tetrahedral). Thus both Zn^{+2} and Cd^{+2} are able to readily form only a σ coordinate covalent bond with a soft Lewis base of the enzyme, such as the sulfhydryl group.

Because all of the metal ions affecting the activity of normal adult human erythrocytic ALAD are borderline or soft Lewis acids it seems reasonable to assume that they are all acting at the same soft Lewis base site of the enzyme with activation or inhibition of the enzyme depending on the relative strengths of the coordination bond formed. The fact that the -SH group, a soft Lewis base, is the most active group found in cells, with coordinate covalent bonding leading to metal mercaptide formation being among the most specific of its reactions (Boyer, 1959), together with the fact that ALAD contains a very large number of -SH groups (Wilson *et al.*, 1972; Shemin, 1972; Tsukamoto *et al.*, 1975; Shemin, 1976) strongly suggests that the soft Lewis base with which these metal ions interact is the -SH group. In addition, the sulfur atom of the -SH group has both empty *d* orbitals that can accept electron pairs from a central ion

and unshared pairs of electrons that can be donated to a central ion in the formation of π coordinate bonds which would, in either case, be strong enough to account for inhibition of the enzyme by metal ions.

Both Zn^{+2} and Cd^{+2} are able to prevent or reverse the *in vitro* inhibition of ALAD induced by the borderline Lewis acid Pb^{+2}. Zn^{+2} is also able to prevent or reverse the *in vitro* inhibition of ALAD induced by the borderline Lewis acid Sn^{+2} but not that induced by the soft Lewis acids Ag^{+} and Hg^{+2}. These results are easily explained if all six ions compete for the same binding sites on the enzyme. The borderline acid-soft base bond formed by Pb^{+2} and Sn^{+2} with the enzyme appears to be strong enough to inhibit the enzyme but weak enough (perhaps due to π bond formation by donation of the electron pairs of the metal ions to the empty *d* orbitals of the sulfide) to permit exchange with sufficiently high concentrations of Zn^{+2} or Cd^{+2}. The soft acid-soft base bond formed by Ag^{+} and Hg^{+2} with the enzyme appears to be strong enough to not only inhibit the enzyme but also (perhaps due to π bond formation by donation of the electron pair of sulfur to the empty *p* orbitals of the metal ions) to prevent exchange with Zn^{+2} no matter how high the concentration. Thus Pb^{+2} and Sn^{+2} may exchange readily with Zn^{+2} and Cd^{+2} but Ag^{+} and Hg^{+2} may not exchange with Zn^{+2} because π bonds that do not contribute to completing a noble gas electronic configuration are more readily broken than those that

do.

The unique ability of Zn^{+2} to both activate ALAD *in vitro* and to prevent or reverse the *in vitro* Pb^{+2}-induced inhibition of ALAD even after a 1.0 hour preincubation with the enzyme emphasizes the importance of not only the divalent, d^{10} characteristics with a characteristic coordination number that gives the metal ion a noble gas electronic configuration and a nonplanar coordination geometry but also the borderline Lewis acid characteristics of a metal ion activator of ALAD. The failure of Cd^{+2} to activate ALAD *in vitro* after even a 0.5 hour preincubation with the enzyme as well as the inability of Zn^{+2} to prevent or reverse the Cd^{+2}-induced inhibition of ALAD is most likely related to the soft Lewis acid characteristics of Cd^{+2} since its other important properties are the same as those of Zn^{+2}. It is possible that on preincubation the kinetics of the Cd^{+2}-enzyme interaction permit Cd^{+2} to assume a 6 coordinate octahedral geometry which allows the soft acid-soft base bond formed between Cd^{+2} and the enzyme to be strengthened (due to π bond formation by donation of the electron pair of sulfur to an empty orbital of Cd^{+2}), resulting in enzyme inhibition. Thus, of all the metal ions studied, the metal ion most likely to function as an *in vivo* activator of normal adult human erythrocytic ALAD and to serve as a possible prophylactic and therapeutic agent in the prevention or treatment of lead poisoning is Zn^{+2}.

If Zn^{+2} is indeed the *in vivo* activator of ALAD, the inhibition of ALAD by Pb^{+2} may be a result of Pb^{+2} replacing Zn^{+2} at its binding sites on the enzyme. This, together with the fact that ALAD loses most, if not all, of its metal ions on purification, would strongly suggest that Zn^{+2} activated ALAD is a metal-enzyme complex and not a metalloenzyme. In order to confirm whether or not ALAD is a Zn^{+2} metal-enzyme complex, the stability constant for the binding of Zn^{+2} to the enzyme must be determined.

2. A CONSIDERATION OF THE EFFECT OF COPPER ON ALAD

All of the metal ions in the present study, with the exceptions of Cu^{+2}, have the stable electronic configurations of a noble gas or one in which the subshells are either filled or half filled. The usual stable electronic configuration of Cu^{+2}, $3\underline{d}^9$, is attributable to the fact that the energies of the $3\underline{d}$ and $4\underline{s}$ orbitals in neutral atoms of the first transition series are quite similar but the $\underline{d}$ orbitals become stabilized relative to the $\underline{s}$ orbitals when atoms have an oxidation state of 2 or more. Therefore, among the elements of the first transition series, the +1 oxidation state is rare regardless of its electronic configuration. Copper is unusual even in this respect because the Cu^{+} state is important due to its stable, $\underline{d}^{10}$, electronic configuration. However, at least in aqueous systems, the Cu^{+2} state is the dominant ionic form because of the greater

lattice and solvation energies and formation constants for its complexes (Cotton and Wilkinson, 1972).

The borderline Lewis acid Cu^{+2} has a square planar coordination geometry so, as expected on the basis of the results obtained for Pb^{+2}, Sn^{+2}, Ag^{+}, and Hg^{+2}, it inhibits erythrocytic ALAD at very low concentrations. Having both a $3\underline{d}^{9}$ valence electronic configuration with its single unpaired electron and a characteristic coordination number of 4, Cu^{+2} is unable to achieve the electronic configuration of a noble gas through σ coordinate bond formation or through accepting electrons into empty $\underline{p}$ orbitals in the formation of a π coordinate bond. Cu^{+2} can, however, donate $\underline{d}$ orbital electrons to the ligand causing inhibition of ALAD that can be prevented or reversed by Zn^{+2}. Thus the inhibition of ALAD by Cu^{+2} and its reversal by Zn^{+2} appear to be very similar to that of both Pb^{+2} and Sn^{+2}.

Of all the metal ions not investigated in the present study, the only other ion that could possibly function as a metal ion activator of ALAD on the basis of the observations of this study is Cu^{+}. Cu^{+} is a soft Lewis base with the same $\underline{d}^{10}$ valence electronic configuration, characteristic coordination number of 4, and tetrahedral coordination geometry as Zn^{+2} and Cd^{+2}. The soft Lewis base nature of Cu^{+} together with its ready oxidation to the powerful ALAD inhibitor Cu^{+2}, however, make this unlikely. On the other hand, cupric copper reacts with mercaptides to give cuprous

copper and disulfide in a reversible reaction that proceeds to the right if the cuprous ion is stabilized by complexation with suitable ligands, the strongest of which is RS^-, in the absence of air (Kolthoff and Stricks, 1951; Klotz et al., 1958; Hemmerich, 1966):

$$2Cu^{+2} + 4RS^- \rightleftharpoons RSSR + CuSR.$$

Reduced thiols have been reported to be necessary for the maximum activity of erythrocytic ALAD, especially after purification of the enzyme, presumably in order to reduce oxidized sulfhydryl groups. However, the possibility exists that the enzyme loses activity due to air oxidation of the essential enzyme activator Cu^+ to the inhibitor Cu^{+2} during the process of purification of ALAD and the in vitro analysis of the enzyme activity and reduced thiols are necessary for maximum activity because they reduce Cu^{+2} to Cu^+. Reduced glutathione may be necessary for the maintainance of in vivo ALAD activity (Moore et al., 1971) for the same reason, maintaining the essential enzyme activator of ALAD, copper, in its reduced form, Cu^+, because the oxidized form, Cu^{+2}, inhibits the enzyme. Lead could, therefore, inhibit ALAD by reducing GSH concentrations, which it is known to do (Shiraishi, 1952; Rubino et al., 1963; Batolska and Marinova, 1970), as a result of which Cu^+ remains oxidized as Cu^{+2} which inhibits the enzyme. The activity of the "lead inhibited" enzyme could then be restored by Zn^{+2} because it replaces Cu^+ with which it is isoelectronic.

These suggestions would explain why copper was the only metal ion found in stoichiometric amounts in beef liver ALAD by Iodice et al. (1958) and why there was a good correlation between ALAD activity and the copper content of the enzyme from U. sphaerogena (Komai and Neilands, 1968).

The absolute requirement of ALAD for Cu^{+2} would be very difficult to demonstrate in light of the fact that Cu^{+} immediately disproportionates to Cu^{+2} and elemental copper upon ionization of its soluble salts in water (Cotton and Wilkinson, 1972). Cuprous acetonitrile perchlorate, $Cu(CH_3CN)_4ClO_4$, prepared according to the method of Hemmerich and Sigwert (1963) as modified by Lonti et al. (1965), is stable toward hydrolysis in the presence of excess acetonitrile (1.0 M), provided the critical pH of 6 has not been exceeded, and could be used in an initial study of Cu^{+} activation of ALAD. Indeed, $Cu(CH_3CN)_4ClO_4$ has been used to reconstitute several Cu^{+}-containing proteins including haemocyanin (Lonti et al., 1965) and cytochrome c oxidase (Nair and Mason, 1967). However, its use in studying ALAD may present certain difficulties such as the low pH and the very high concentrations of the organic solvent acetonitrile that must be maintained, especially in light of the fact that very much smaller concentrations of another organic solvent, ethanol, inhibit ALAD (Moore et al., 1971). These difficulties will have to be carefully considered and, hopefully, somehow circumvented.

3. THE IN VIVO INVESTIGATION OF THE EFFICACY OF ZINC AS A PROPHYLACTIC OR THERAPEUTIC AGENT IN LEAD POISONING

In 1972 Willoughby and associates reported the results of a study in which groups of young growing horses were fed an alfalfa-grass hay mixture plus increasing amounts of a basal ration. The basal ration was either a control diet (containing 4.0 ppm Pb^{+2} and 180 ppm Zn^{+2}), a diet containing toxic amounts of Pb^{+2} only (800 ppm Pb^{+2} as $PbCO_3$ and 170 ppm Zn^{+2}), a diet containing toxic amounts of Zn^{+2} only (18 ppm Pb^{+2} and 5400 ppm Zn^{+2} as ZnO), or a diet containing the same toxic amounts of both Pb^{+2} and Zn^{+2} as the diets containing only toxic amounts of Pb^{+2} or Zn^{+2} over a 23 to 38 week period. They found that toxic amounts of Zn^{+2} interfere with the uptake of Pb^{+2} in areas of active bone formation and result in higher hepatic and renal Pb^{+2} without these high levels being associated with or causing impaired neurologic function. Therefore, toxic amounts of Zn^{+2} appeared to prevent the development of pharyngeal and laryngeal paralysis, clinical signs of lead poisoning in the young growing horse, but the exact mechanism was unknown.

In another study Thawley et al. (1977) fed rats lead (0.5%), zinc (0.63%), cadmium (90 ppm), and a control diet alone and in combination for 42 days with two levels of calcium (0.1 and 0.9%) and three levels of vitamin D (0, 2000. and 50,000 IU/kg of feed). They noted a reduction in urinary ALA excretion and lowered blood and tissue lead levels

in lead fed rats that were also fed excess zinc. A reduction in urinary ALA excretion was also observed in lead fed rats fed excess cadmium in a low vitamin D diet. These authors, however, were unable to determine whether the mechanism for the antagonism of the toxic effects of lead by zinc was at the level of gastrointestinal absorption or at the level of metabolic interference within the hemopoietic system.

Thawley and co-workers (1978) also conducted a study in which groups of rats were fed a control diet, a diet containing 0.6% zinc, and a diet containing 0.5% lead and 0.6% zinc for six weeks. Two additional groups of rats were used, one group receiving the basal diet and intraperitoneal injections of 0.25 ml of a 20% ZnO suspension on the first, fourteenth, and twenty-eighth days of the six week experimental period and the others receiving a diet containing 0.5% lead and the same intraperitoneal injections of a 20% ZnO suspension. They found that the oral or intraperitoneal administration of zinc resulted in the reduction of urinary ALA excretion in lead intoxicated rats to nearly normal levels without any significant reduction in blood lead levels or any alleviation of the anemia of lead poisoning. This led these investigators to conclude that the effect of zinc is at a physiologic level beyond gastrointestinal absorption, presumably reactivating ALAD inhibited by lead or preventing inhibition of ALAD by lead. These investigators

also found that Pb^{+2} administration caused a significant decrease in blood Zn^{+2} levels.

Haeger-Aronsen and associates (1976) recently investigated the effect of lead (as lead acetate), zinc (as zinc sulfate), and lead and zinc together in the rabbit when injected subcutaneously on the back. One group of animals received 63 mg Zn^{+2}/kg on day 0 and 200 mg Zn^{+2}/kg on day 28; another group of rabbits received 18 mg Pb^{+2}/kg on day 0 and 50 mg Zn^{+2}/kg on day 28; a third group of rabbits received 25 mg Pb^{+2}/kg on day 0 while the fourth group received 25 mg Pb^{+2}/kg on day 0 and 200 mg Zn^{+2}/kg on day 35. They found that zinc has a strong activating effect on ALAD in vivo, nearly eliminating the inhibitory effect of lead.

In 1976 Cerklewski and Forbes reported the results of an investigation of the influence of dietary zinc on the toxicity of dietary lead in the young male rat. Nine groups of rats were fed a semipurified diet containing 8, 35, or 200 ppm zinc (as zinc carbonate) alone or in combination with 0, 50, or 200 ppm lead (as lead acetate) over a seven week period. They found that as dietary zinc was increased the severity of lead toxicity decreased. The evidence for this decrease in lead toxicity was: a decreased concentration of lead in blood, liver, kidneys, and tibias; a decreased excretion of urinary ALA; a decreased accumulation of FEP; a decreased inhibition of kidney ALAD. In a second experiment they investigated the effect of zinc injected

intraperitoneally (as zinc sulfate) on the toxicity of dietary lead and found it afforded no protection. They therefore concluded that the protective effect of zinc against lead toxicity is primarily the result of an inhibition of the intestinal absorption of lead.

In a similar study Cerklewski and Forbes (1977) examined the effect of dietary copper on the toxicity of dietary lead in the young male rat. Six groups of rats were fed a semipurified diet containing 1, 5, or 20 ppm copper (as cupric chloride) alone or in combination with 0 or 200 ppm lead (as lead acetate) over a four week period. They found that as dietary copper increased so did the severity of the lead toxicity. The evidence for the increased lead toxicity was an increased lead concentration in the kidney and a two- to threefold increase in the excretion of urinary ALA. These investigators hypothesize that the increasing dietary copper concentration may have a modifying effect on other trace elements (such as zinc), producing more favorable conditions for the development of lead toxicity.

A recent article (Thomasino et al., 1977) reports the interesting case of a lead intoxicated 37 year old male with an extremely high blood lead level (260 μg/100 ml of whole blood) studied before and after treatment with Na_2(Ca-EDTA) and then before and after oral administration of zinc sulfate. Immediately after 5 days of daily parenteral doses

of 500 mg Na_2(Ca-EDTA) the blood lead level was 100 µg/100 ml of whole blood and continued to fall until, at 49 days after the onset of chelation therapy, it was 55 µg/100 ml of whole blood. Serum zinc concentrations fell from 146 to 68 µg/100 ml during chelation therapy while 24 hour urinary zinc excretion rose dramatically, reaching a peak of nearly 7000 µg/24 hours during the last day of therapy which was nearly five times higher than the highest prechelation value. During chelation therapy more than 27,281 µg of Zn^{+2} was excreted which was nearly four times more than the 7700 µg of Pb^{+2} excreted. Erythrocytic ALAD activity was 20% of the control value prior to chelation therapy, declined to 12.2% of control during chelation, and rose to 25.5% of control on the day after chelation was discontinued. Thirty-six days after chelation therapy ended 100 µg of zinc sulfate was administered orally to the patient three times a day for five days. The zinc sulfate therapy had little effect on the serum Zn^{+2}, blood Pb^{+2}, urinary Zn^{+2}, or urinary Pb^{+2} concentrations. There was, however, a significant increase in erythrocytic ALAD activity to 35% of the control value as a result of the Zn^{+2} therapy. On the basis of these results the authors concluded that Zn^{+2} may play a protective role in lead toxicity and Zn^{+2} supplementation may be a useful adjunct to chelation therapy for lead toxicity. Unfortunately these results are not as clear cut as they might have been since the biggest decrease (50%)

in both blood lead and serum zinc were observed as a result of eight days of hospitalization prior to the onset of chelation therapy. The decreased blood lead is most likely due to a discontinuation of exposure to the environment of the lead battery factory where the subject was employed while the decreased serum zinc may be due to a discontinuation of exposure to the several pints of whiskey he consumed daily for several years.

Nonetheless, the suggestion that Zn^{+2} supplementation may be useful adjunct to chelation therapy for lead toxicity or a useful prophylactic agent for preventing lead toxicity seems reasonable. The reasons for this bear reviewing:

a. Pb^{+2} inhibits erythrocytic ALAD
b. Pb^{+2} causes a significant decrease in the serum Zn^{+2} concentration
c. Na_2(Ca-EDTA) therapy results in a marked increase in the urinary excretion of Zn^{+2} and a corresponding fall in serum Zn^{+2} levels
d. Zn^{+2} deficiency results in a marked decrease in erythrocytic ALAD activity
e. inhibition of ALAD contributes significantly to the anemia of lead poisoning
f. inhibition of ALAD results in a dramatic accumulation of the substrate ALA in the serum and various organs

g. the increased levels of ALA may be responsible for some of the neurologic, gastrointestinal, and cardiovascular symptoms of lead poisoning
h. Zn^{+2} activates and prevents or reverses Pb^{+2}-induced inhibition of erythrocytic ALAD *in vitro*
i. toxic levels of Zn^{+2} prevent or reverse the toxic effects of Pb^{+2} *in vivo* at the level of gastrointestinal absorption, at the level of interference with the hemopoietic system, or at both levels
j. Zn^{+2} can be administered in very large doses without toxic effects (National Research Council Subcommittee on Zinc, 1979).

Before Zn^{+2} supplementation is used as a prophylactic agent or therapeutic adjunct in the prevention or treatment of lead poisoning it is desirable to understand whether Zn^{+2} primarily prevents the gastrointestinal absorption of Pb^{+2} or prevents or reverses the Pb^{+2}-induced inhibition of ALAD so a protocol for Zn^{+2} supplementation can be established for each use.

In determining the site of Zn^{+2} interference with lead toxicity a most important consideration is the choice of an animal model. While no single species is an exact model for the effect of lead in man, the rabbit is among the few animals in which lead poisoning interferes with porphyrin metabolism as it does in man (Hass *et al.*, 1964; Scharding and Oehme, 1973) and the location and sequence of the development

of renal pathology resemble those of man (Hass et al., 1964; Macadam, 1969; Scharding and Oehme, 1973). In addition, the activity of rabbit erythrocytic ALAD is one of the few erythrocytic ALAD activities that is at least as high as that of man (Abdulla et al., 1978a). The rabbit, therefore, seems to be a logical choice as an animal model for this study.

In order to evaluate the efficacy of Zn^{+2} as a prophylactic agent for the prevention of lead poisoning or as a therapeutic agent for the treatment of lead poisoning the following protocol is proposed and could be modified as the need arose. Fifty-six young adult male New Zealand white rabbits should be divided into seven groups of eight and fed a control diet, a control diet supplemented with zinc acetate, a control diet supplemented with lead acetate, or a control diet supplemented with both Pb^{+2} and Zn^{+2}. The daily lead dose should be chosen because it is sublethal and the Zn^{+2} dose should be chosen so as to provide a molar concentration of Zn^{+2} that is equal to or twice the molar concentration of the sublethal dose of lead but is itself subtoxic. The seven groups could be:

a. animals fed a control diet
b. animals fed 20 mg Pb^{+2}/kg/day in a control diet for 24 weeks
c. animals fed 6.3 mg Zn^{+2}/kg/day in a control diet for 24 weeks

d. animals fed 20 mg Pb^{+2}/kg/day and 6.3 mg Zn^{+2}/kg/day in a control diet for 24 weeks

e. animals fed 20 mg Pb^{+2}/kg/day in a control diet for the first 12 weeks and a control diet for the last 12 weeks

f. animals fed a control diet for the first 12 weeks and 6.3 mg Zn^{+2}/kg/day in a control diet for the last 12 weeks

g. animals fed 20 mg Pb^{+2}/kg/day in a control diet for the first 12 weeks and 6.3 mg Zn^{+2}/kg/day in a control diet for the last 12 weeks

At the end of the experimental period the animals should be weighed and 24 hour urines collected. The animals can then be sacrificed, blood collected in the appropriate Vacutainer tubes, and the following determined on the blood and urine:

a. 24 hour urinary output

b. serum and urinary creatinine

c. complete blood count

d. reticulocyte percent

e. erythrocytic ALAD activity, unactivated by Zn^{+2} and activated by Zn^{+2} so the ratio can be determined

f. serum and urinary ALA

g. whole blood and urinary lead

h. serum and urinary zinc

i. serum copper

j. serum iron

(It is necessary to determine serum copper because zinc is reported to act as a copper antagonist (Hill, 1976) and serum iron in order to rule out iron deficiency anemia.) Upon analyzing these results using an analysis of variance one should be able to determine at which site Zn^{+2} antagonizes the toxic effects of Pb^{+2}.

In addition to the above measurements at the end of the above experiment it might be interesting to do some histology on at least the kidney and perhaps also the liver and bone marrow in order to determine whether or not Zn^{+2} prevented any lead-induced change in their histology.

Once it has been determined at which site or sites Zn^{+2} is acting, its efficacy as a prophylactic agent or therapeutic adjunct should be apparent. If Zn^{+2} is indeed potentially valuable as a therapeutic adjunct, further studies can be conducted in order to determine the proper doses of Zn^{+2} to be used in conjunction with Na_2(Ca-EDTA) and the times at which the doses should be administered with respect to the times of administration of the chelating agent. If Zn^{+2} is indeed potentially valuable as a prophylactic agent, further studies can be conducted in order to determine the proper doses of Zn^{+2} to be used for the various routes and levels of lead exposure.

CHAPTER VI

SUMMARY

CHAPTER VI

SUMMARY

A correlation between blood reticulocyte percent and the activity of erythrocytic ALAD in the developing rat has been established during the progression from a state of macrocytic hypochromic anemia at birth to a normocytic normochromic state at puberty and, finally, to a state just prior to adulthood at which the reticulocyte percent had stabilized. Both reticulocyte percent and erythrocytic ALAD activity were found to decrease with age, rapidly at first until a normocytic normochromic state was reached at puberty and then more slowly until just before adulthood when both plateaued. A direct, linear correlation between erythrocytic ALAD activity and blood reticulocyte percent was found with a P value of less than 0.001. These findings should be carefully considered when using the rat as a model for lead poisoning and, possibly, for other disorders of heme synthesis.

When establishing reference values for the ALAD activity of normal, healthy, nonmedicated adult male and female volunteers a highly significant difference was found between the mean male ALAD activity of 44.2 mU/ml RBC and the mean female ALAD activity of 48.2 mU/ml RBC, with a P value of less than 0.01. While the biological significance of this

difference is uncertain at the present time since it has been suggested that there is excess enzyme present in the body, it should, nonetheless, be considered when establishing reference values for the erythrocytic ALAD assay.

It was the major objective of this work to determine, in a dose-response fashion, the in vitro effects of Na^{+}, Mg^{+2}, Al^{+3}, Mn^{+2}, Cu^{+2}, Ag^{+}, Zn^{+2}, Cd^{+2}, Hg^{+2}, Ga^{+3}, In^{+3}, Pb^{+2}, Sn^{+2}, and Sn^{+4} on normal erythrocytic ALAD and on an enzyme 50% inhibited by Pb^{+2} while at the same time examining the ability of a maximally stimulatory concentration of an activator metal ion discovered on screening, Zn^{+2}, to prevent or reverse the effects of these metal ions. As a result of this work several important conclusions were reached concerning the physical and chemical properties associated with the ability of a metal ion to activate or inhibit human erythrocytic ALAD. All hard Lewis acids, regardless of valence electronic configuration or oxidation state, were found to have no significant effect on ALAD activity except at very high concentrations. Divalent zinc and cadmium, borderline or soft Lewis acids having characteristic coordination numbers that give them the electronic configuration of noble gases, were the only ions found to activate erythrocytic ALAD and to prevent or reverse the Pb^{+2}-induced inhibition of the enzyme. Zn^{+2} could prevent or reverse the in vitro inhibition of ALAD by low concentrations of borderline Lewis acids having characteristic

coordination numbers that give them the electronic configuration of noble gases and a planar coordination geometry that allow π bonding through donating electrons into an empty d orbital of a ligand. Zn^{+2} could not, however, prevent or reverse the inhibition of ALAD induced by low concentrations of soft Lewis acids having characteristic coordination numbers that do not give them the electronic configuration of noble gases and a planar coordination geometry that allows π bonding through accepting electrons from a ligand into an empty p orbital. On preincubation with the enzyme for as little as one-half hour the soft Lewis acid Cd^{+2} only inhibited ALAD but the borderline Lewis acid Zn^{+2} could still activate ALAD after preincubation with the enzyme for as long as four hours. These results suggest that the metal ions are affecting the activity of ALAD by an interaction with the sulfhydryl groups of enzyme. These results also suggest that Zn^{+2} is possibly the *in vivo* metal ion activator of the metal-enzyme complex ALAD but do not rule out the possibility that it is Cu^{+} and not Zn^{+2} that is the activator of ALAD. It is proposed that Zn^{+2} be used as a prophylactic agent or therapeutic adjunct to prevent or reverse the Pb^{+2}-induced inhibition of ALAD and the resulting anemia and possibly toxic accumlation of the substrate ALA.

CHAPTER VII

FIGURES

FIGURE 1. The biosynthesis of heme. A = acetic, P = propionic, M = methyl, and V = vinyl. For the other abbreviations and a discussion of the pathway see the text.

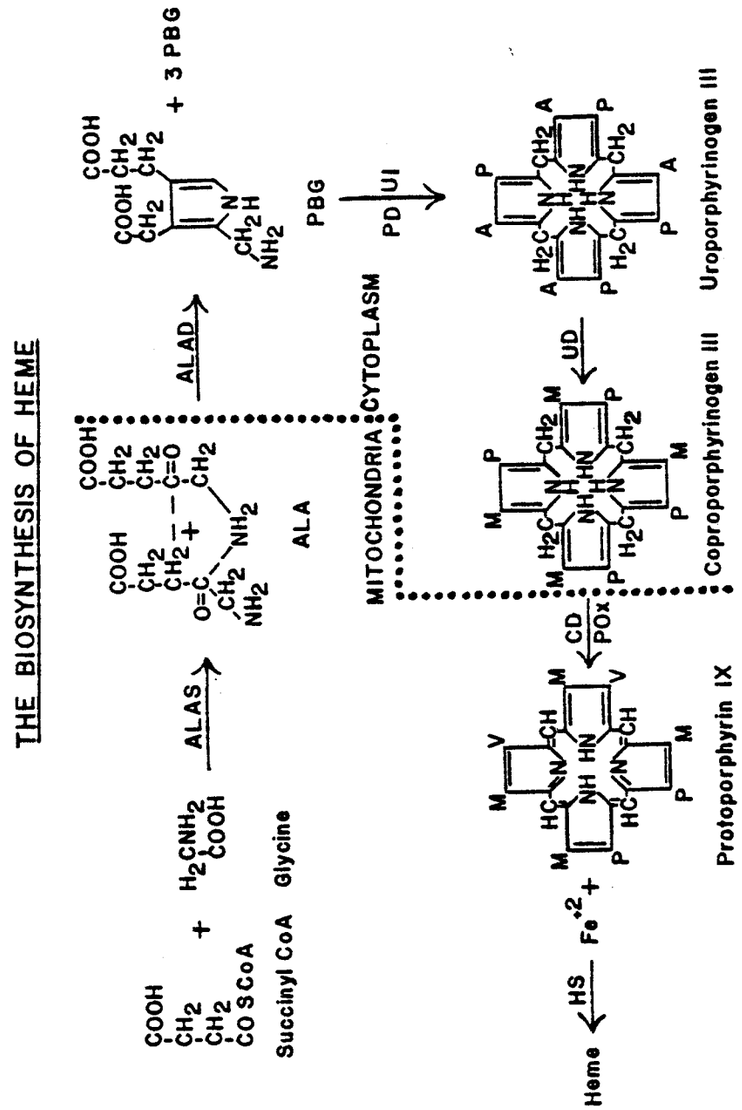

FIGURE 1

FIGURE 2. The procedure for the determination of erythrocytic ALAD activity using an aliquot of heparinized whole blood.

PROCEDURE FOR ASSAY OF ERYTHROCYTIC ALAD ACTIVITY IN WHOLE BLOOD

1. Collect blood into heparinized tube and immediately place tube in an ice-bath.

   (heparinized blood samples can also be stored in a refrigerator at 4 °C up to 24 hrs without loss of ALAD activity)

2. Add 100 microliters of whole blood to 1.5 ml of aqueous 0.2% Triton X-100 in a 16 x 125 mm test-tube, gently vortex and warm to 37 °C for 5 min.

3. Add 1.0 ml of 0.01 M ALA·HCl in 0.25 M dibasic sodium phosphate/citric acid buffer at pH 6.7 also warmed to 37 °C, gently vortex, cover tube and incubate for 1 hr at 37 °C.

4. Stop reaction by the addition of:

   1) 1.0 ml 10% TCA containing 0.02 M N-ethylmaleimide
   2) 0.05 ml saturated cupric sulfate solution

   mix and let stand for 5 min and then centrifuge at 600 x g for 10 min at room temp.

5. Withdraw a 2.0 ml aliquot of the supernatant fluid, add 2.0 ml of modified Ehrlich's reagent (2 N with respect to PCA) and allow 15 min for color development.

6. Read the optical density at 555 nm using a 1.0 cm light path cuvet in a colorimeter set to 100% transmission with a reagent blank in which water is substituted for blood.

FIGURE 2

FIGURE 3. The definition of a milliunit of erythrocytic ALAD activity and the method of calculating the erythrocytic ALAD activity of whole blood.

CALCULATION OF ERYTHROCYTIC ALAD ACTIVITY IN WHOLE BLOOD

Erythrocytic ALAD activity is expressed as mU/ml packed red cells

ALAD mU definition: 1 mU is that amount of enzyme which will catalyze the transformation of 1 mmol of ALA to PBG per min at pH 6.7 at 37 °C

$$\text{ALAD mU/ml RBC} = OD_{60} \times \frac{100}{HCT} \times K$$

OD_{60} = optical density after 1 hr at 37 °C

HCT = microhematocrit as % packed cell volume

K = constant dependent on aliquot of blood used for assay

and

$$K = \frac{3.65 \times 2}{0.1 \times 60} \times \frac{1}{0.060} \times 2 = 40.56$$

where 3.65 x 2 represents total reaction volume x dilution factor for addition of equal volume of modified Ehrlich's reagent to supernatant fluid

0.1 represents ml of blood used for assay

60 represents incubation time in min

0.060 represents the optical density of a final concentration of 1 mmol/ml of PBG experimentally determined under the conditions of the assay

2 represents factor based on production of 1 mol of PBG from 2 mol of ALA

FIGURE 3

FIGURE 4. The procedure for the determination of erythrocytic ALAD activity using an aliquot of heparinized whole blood as modified for <u>in vitro</u> metal ion activation and inhibition studies.

PROCEDURE FOR ASSAY OF ERYTHROCYTIC ALAD ACTIVITY IN WHOLE BLOOD

1. Collect blood into heparinized tube and immediately place tube in an ice-bath.

   (Heparinized blood samples can also be stored in a refrigerator at 4 C up to 24 hrs without loss of ALAD activity.)

2. Add 100 microliters of whole blood to 1.3 ml of aqueous 0.2% Triton X-100 in a 16 x 125 mm test-tube, gently vortex and warm to 37 C for 5 min.

3. Immediately add 100 microliters of aqueous 0.2% Triton X-100 and/or 100 microliters of aqueous 0.2% Triton X-100 inhibitor solution and/or 100 microliters of aqueous 0.2% Triton X-100 activator solution.

4. Immediately add 1.0 ml of 0.01 M ALA·HCl in 0.25 M dibasic sodium phosphate/citric acid buffer at pH 6.7 also warmed to 37 C, gently vortex, cover tube and incubate for 1 hr at 37 C. (Final incubation volume of 2.6 ml.)

5. Stop reaction by the addition of:

   1) 1.0 ml 10% TCA containing 0.02 M N-ethylmaleimide
   2) 0.05 ml saturated cupric sulfate solution

   mix and let stand for 5 min and then centrifuge at 600 x g for 10 min at room temp.

6. Withdraw a 2.0 ml aliquot of the supernatant fluid, add 2.0 ml of modified Ehrlich's reagent (2 N with respect to PCA) and allow 15 min for color development.

7. Read the optical density at 555 nm using a 1.0 cm light path cuvet in a colorimeter set to 100% transmission with a reagent blank in which water is substituted for blood.

FIGURE 4

FIGURE 5. The variation of various hematologic indices, including hemoglobin, hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH), with the age of the male albino rat. Each point represents the average of results obtained from three animals.

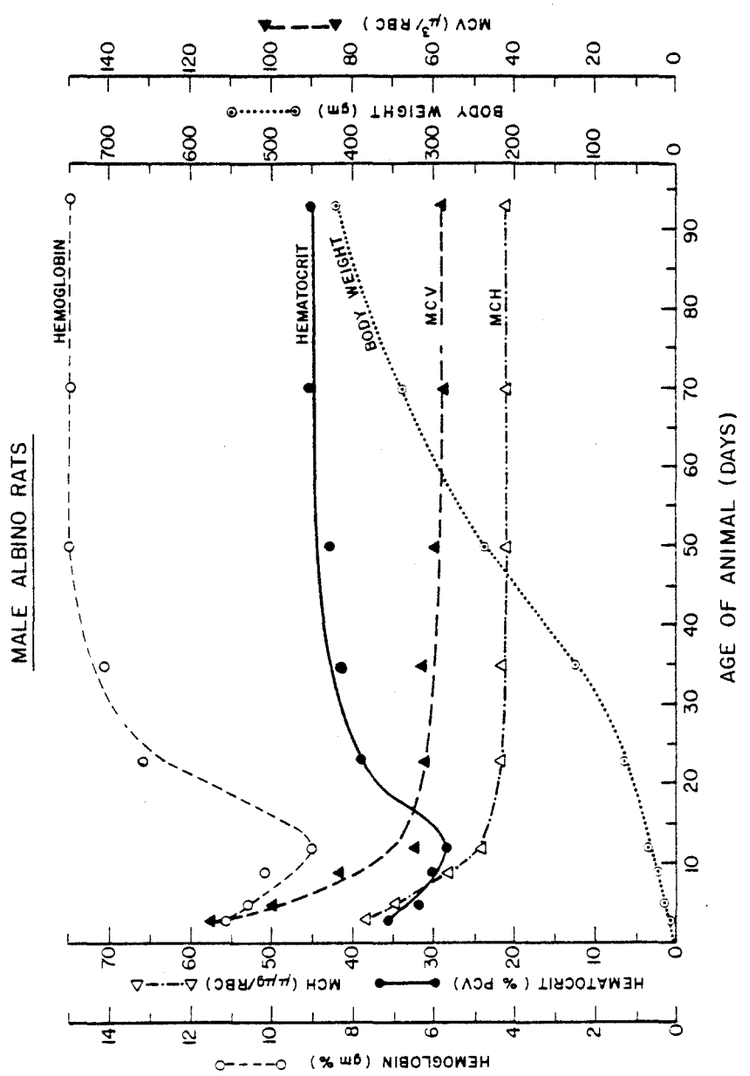

FIGURE 5

FIGURE 6. The variation of blood reticulocyte percent with the age of the male albino rat. Each point represents the average reticulocyte percent per 1000 erythrocytes counted for each of three animals.

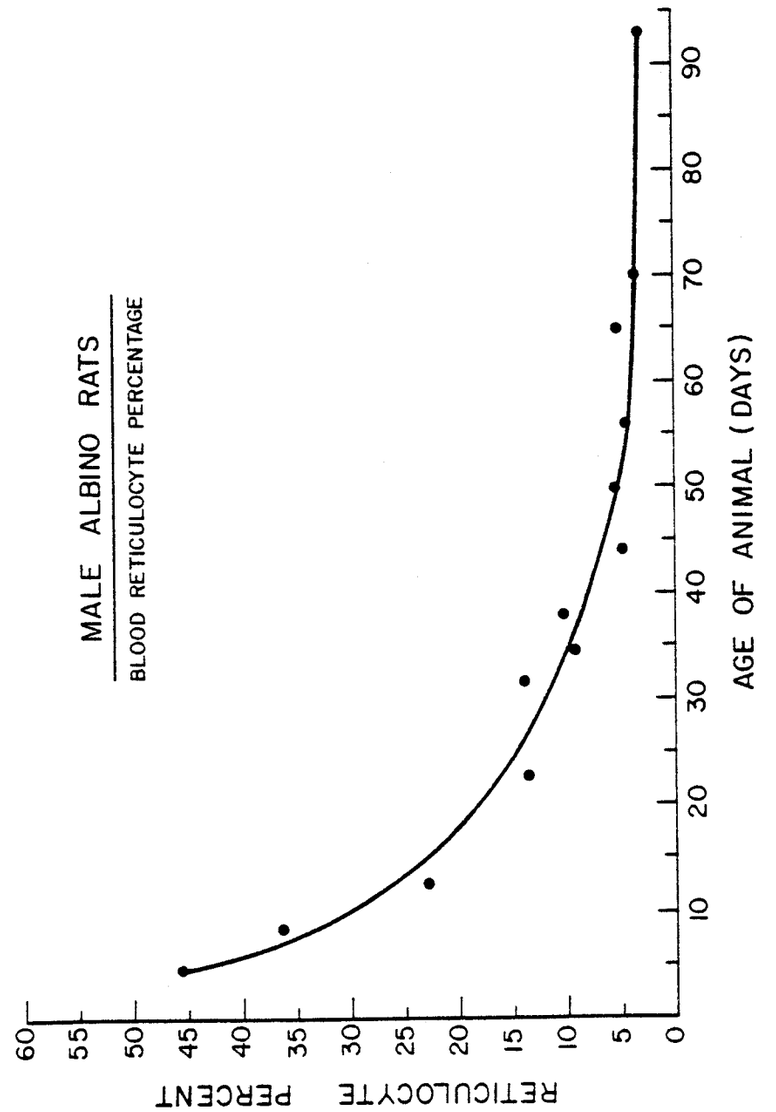

FIGURE 6

FIGURE 7. The variation of erythrocytic ALAD activity with the age of the male albino rat. Each point represents the average of results obtained from three animals.

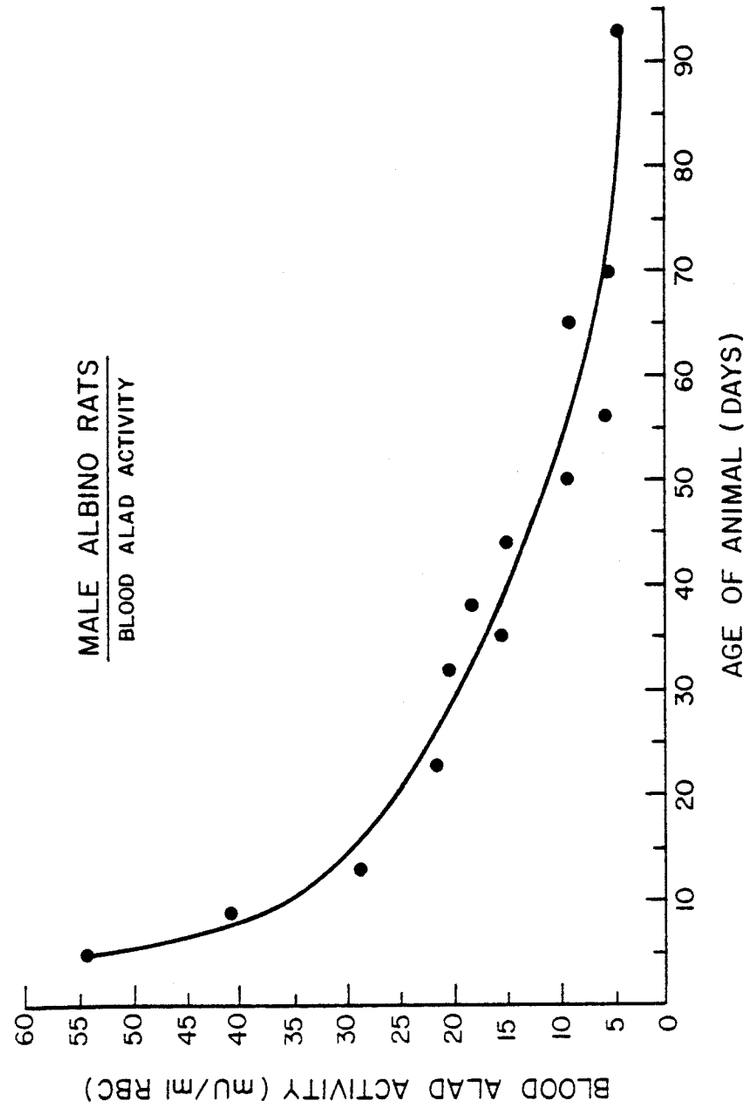

FIGURE 7

FIGURE 8. The relationship between erythrocytic ALAD activity and blood reticulocyte percent. Each point represents the determination of ALAD activity and reticulocyte percent in each of 27 individual male albino rats studied. The correlation coefficient (r) was determined to be 0.967 with a P value of less than 0.001.

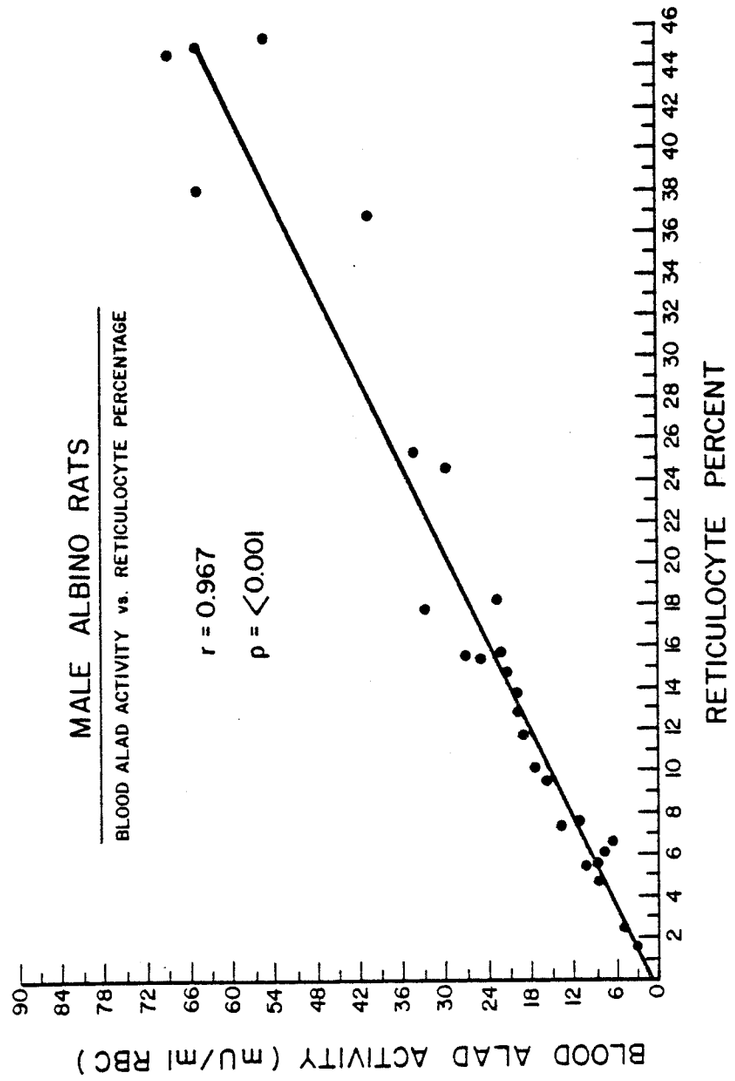

FIGURE 8

FIGURE 9. The concentration curve for the effect of Cu^{+2} on normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

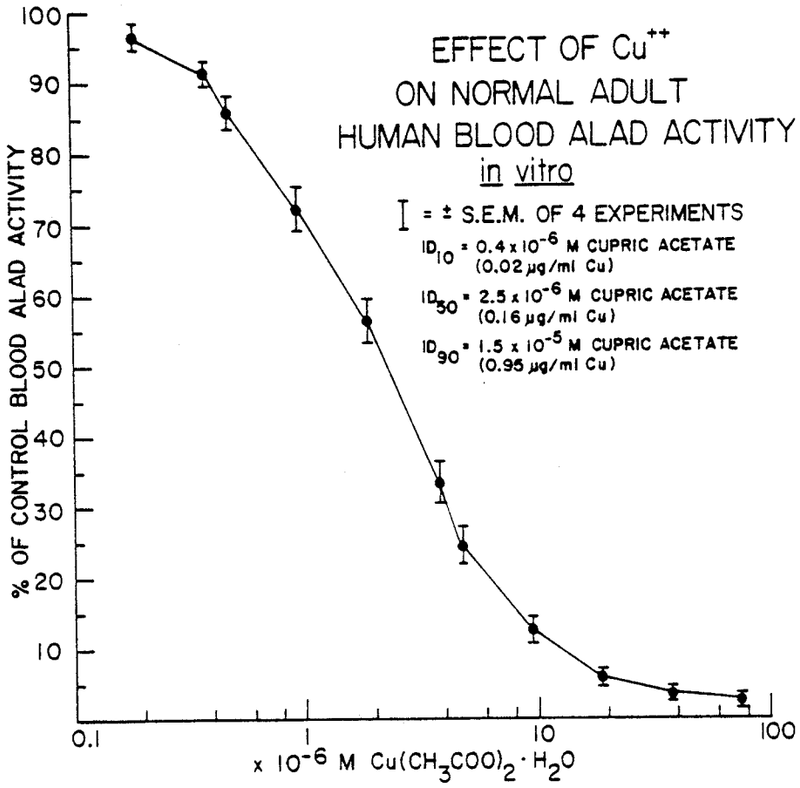

FIGURE 9

FIGURE 10. The effect of various concentrations of Pb^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2} on normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

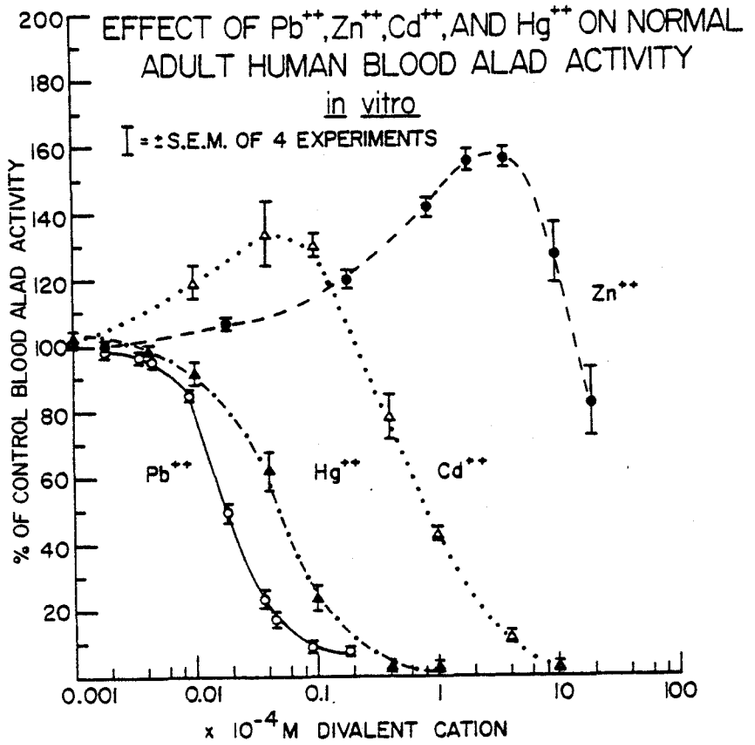

FIGURE 10

FIGURE 11. The effect of zinc acetate on lead acetate-induced inhibition of normal adult human erythrocytic ALAD in vitro. Lead and zinc were added to the hemolysates immediately prior to the 1.0 hour incubation with ALA at 37°C. Each point represents the mean ± S.E.M. of results obtained for 11 individuals.

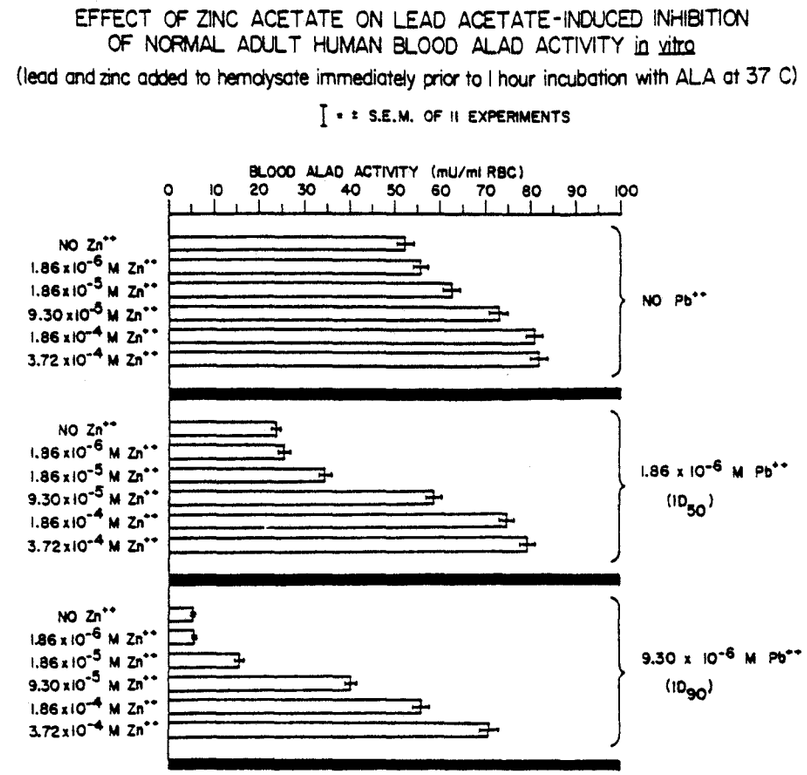

FIGURE 11

FIGURE 12. The effect of zinc acetate on copper acetate-induced inhibition of normal adult human erythrocytic ALAD *in vitro*. Copper and zinc were added to the hemolysates immediately prior to the 1.0 hour incubation with ALA at 37°C. Each point represents the mean ± S.E.M. of results obtained for 6 individuals.

EFFECT OF ZINC ACETATE ON COPPER ACETATE-INDUCED INHIBITION OF NORMAL ADULT HUMAN BLOOD ALAD ACTIVITY *in vitro*
(copper and zinc added to hemolysate immediately prior to 1 hour incubation with ALA at 37 C)

I • ± S.E.M. OF 6 EXPERIMENTS

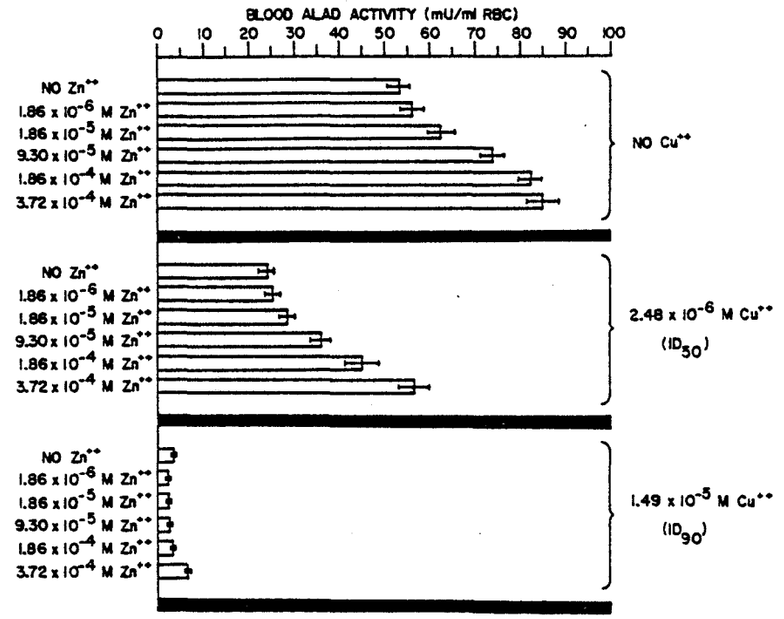

FIGURE 12

FIGURE 13. The concentration curves for the effect of Zn^{+2} of ID_{50} Pb^{+2}- and on ID_{50} Cu^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 11 or 6 individuals, as indicated on the graph.

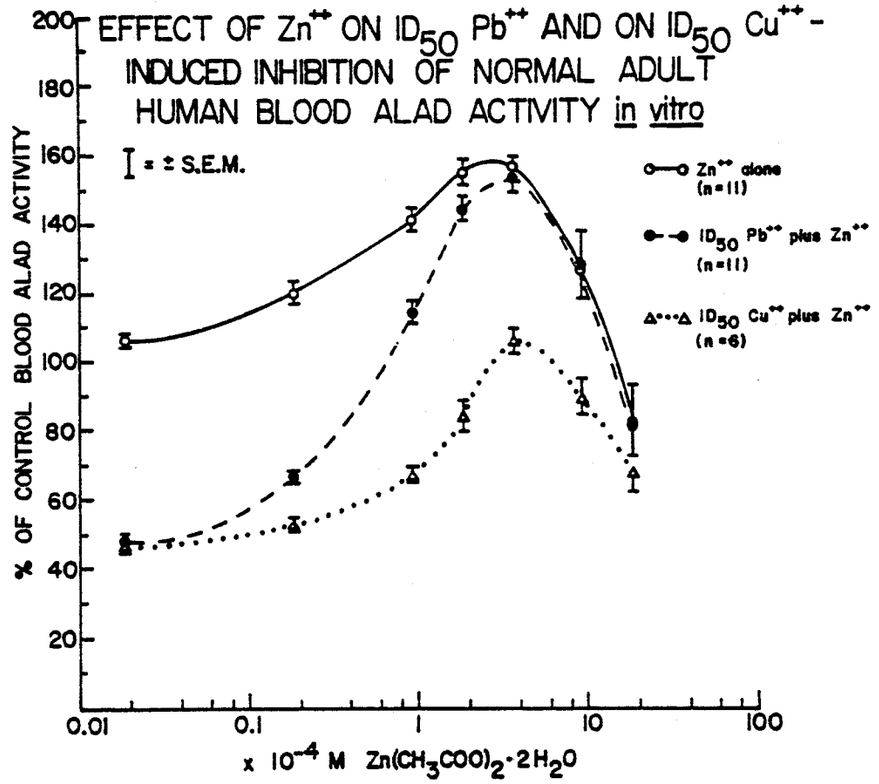

FIGURE 13

FIGURE 14. The concentration curves for the effect of Zn^{+2} on ID_{90} Pb^{+2}- and on ID_{90} Cu^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 11 or 6 individuals, as indicated on the graph.

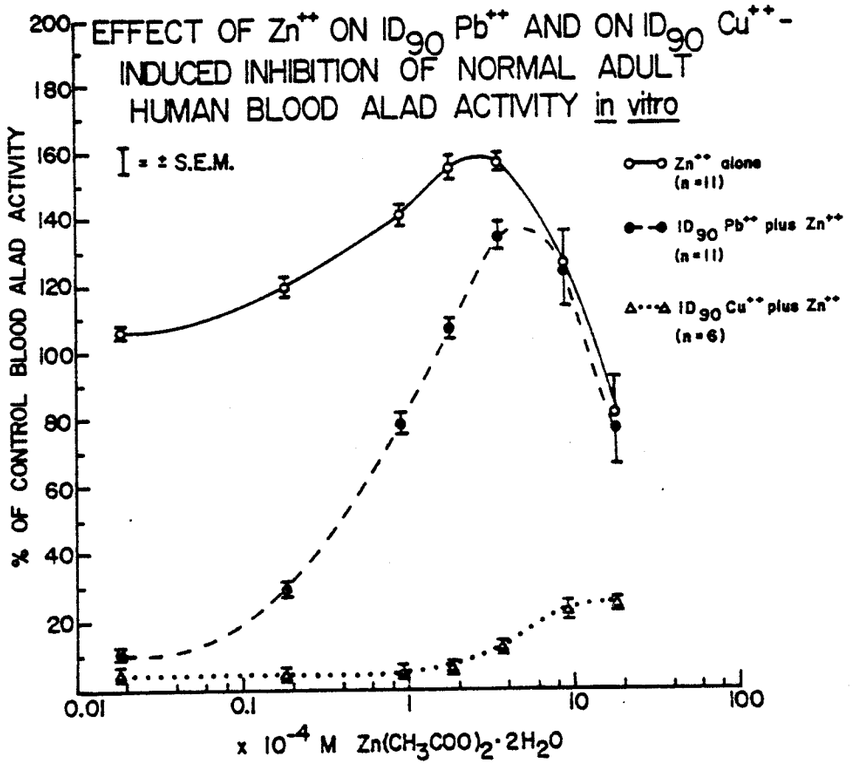

FIGURE 14

FIGURE 15. The effect of various concentrations of Pb^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2} on normal, ID_{50} Pb^{+2} inhibited, and SD_{100} Zn^{+2} stimulated adult human erythrocytic ALAD activity *in vitro*. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

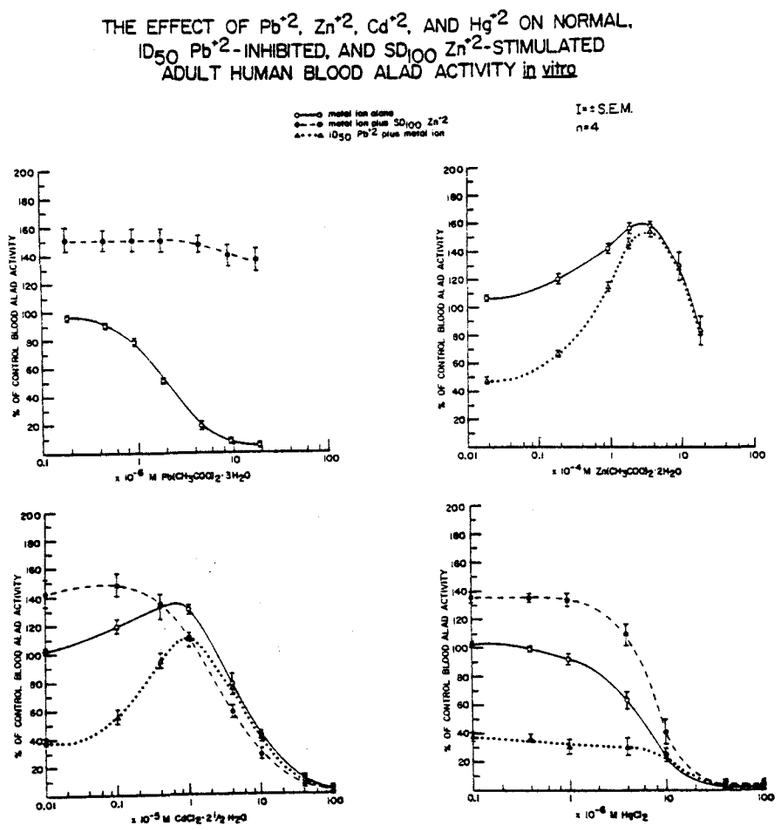

FIGURE 15

FIGURE 16. The concentration curves for the effect of Na^{+}, Mg^{+2}, Al^{+3}, and Mn^{+2} on normal, ID_{50} Pb^{+2} inhibited, and SD_{100} Zn^{+2} stimulated adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

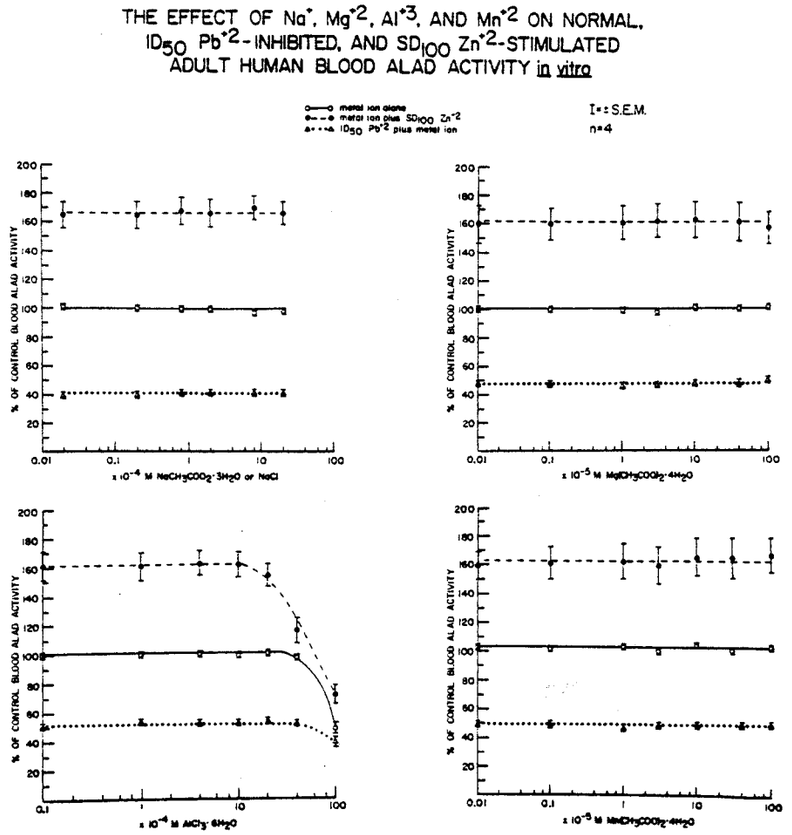

FIGURE 16

FIGURE 17. The effect of various concentrations of Ag^{+} on normal, ID_{50} Pb^{+2} inhibited, and SD_{100} Zn^{+2} stimulated adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

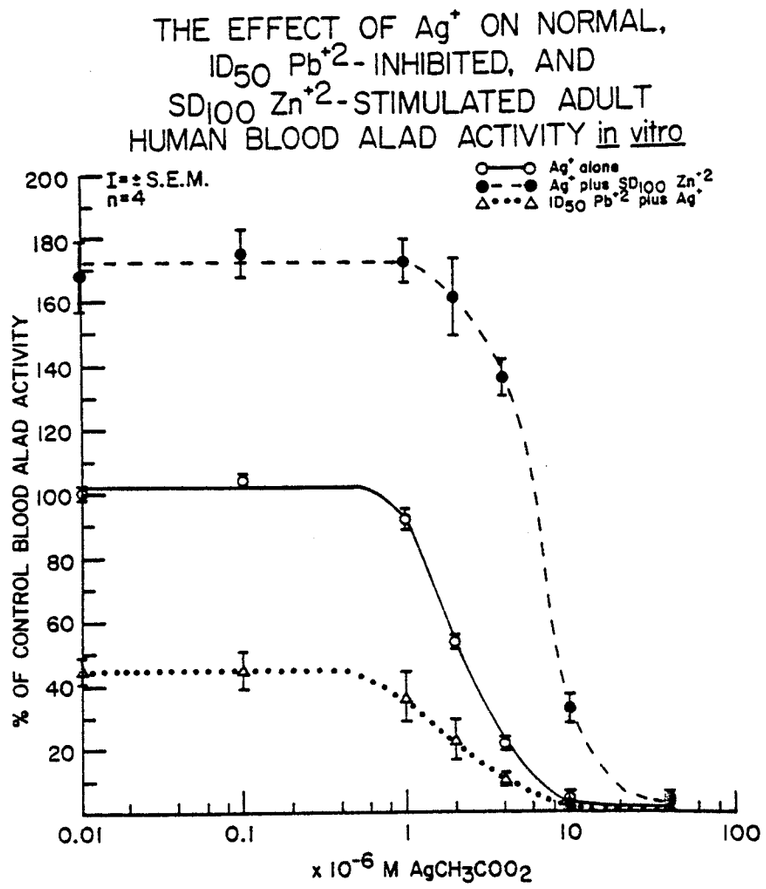

FIGURE 17

FIGURE 18. The concentration curves for the effect of Ga^{+3} and In^{+3} on normal, ID_{50} Pb^{+2} inhibited, and SD_{100} Zn^{+2} stimulated adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

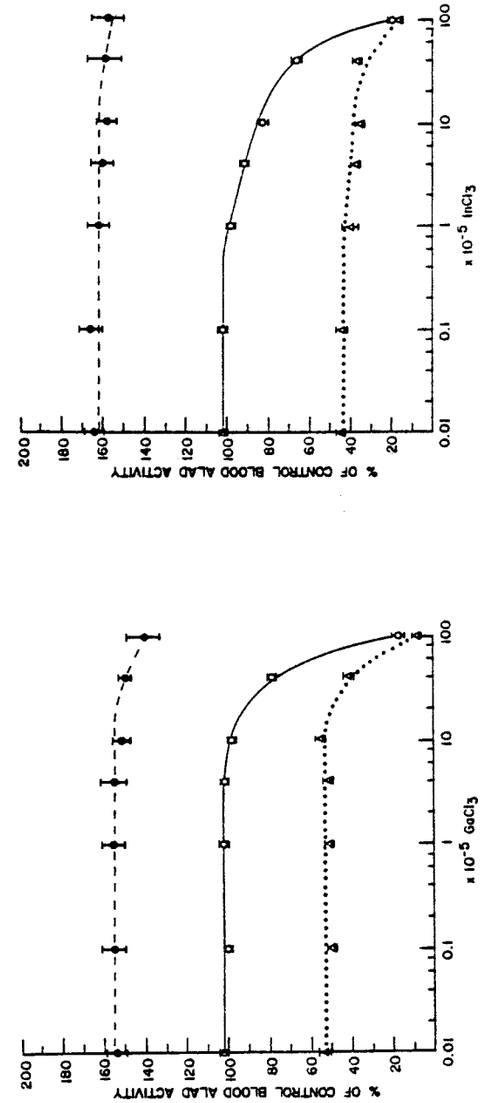

FIGURE 18

FIGURE 19. The concentration curves for the effect of Sn^{+2} and Sn^{+4} on normal, ID_{50} Pb^{+2} inhibited, and SD_{100} Zn^{+2} activated adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

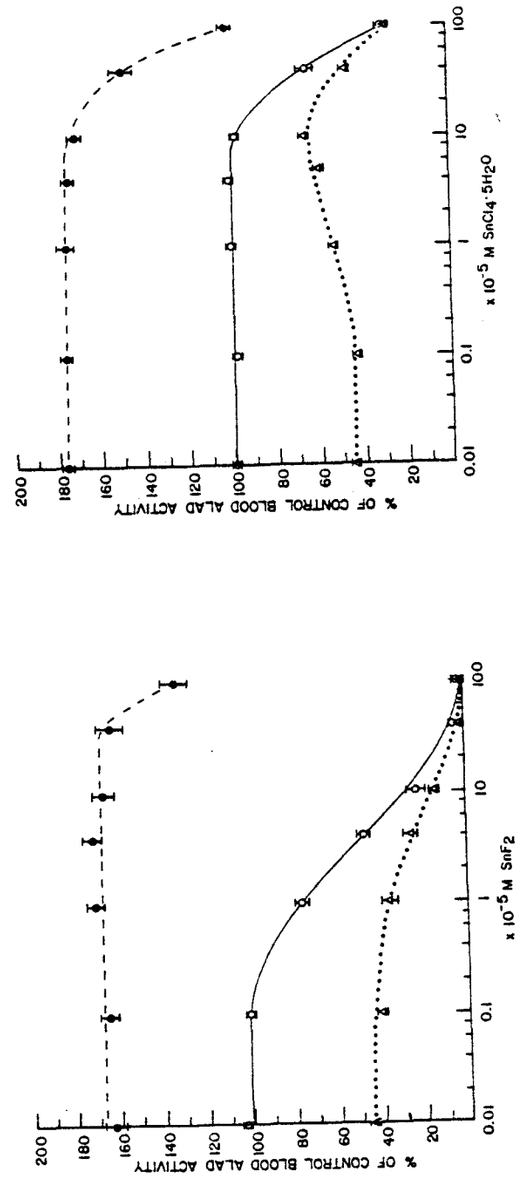

FIGURE 19

FIGURE 20. The effect of 1.0 hour preincubation with Pb^{+2} and Hg^{+2} on Pb^{+2}- and Hg^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

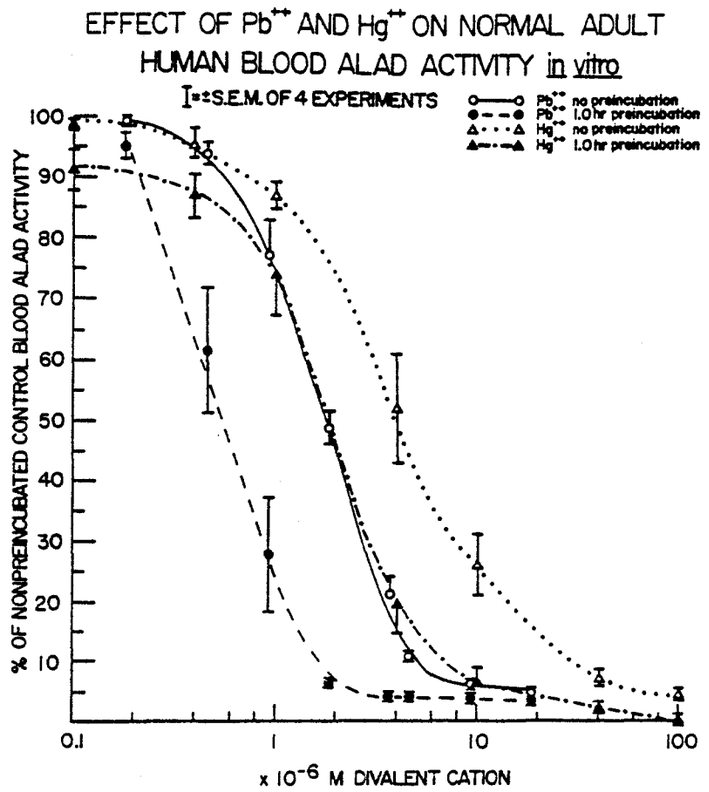

FIGURE 20

FIGURE 21. The effect of 1.0 hour preincubation with Zn^{+2} and Cd^{+2} on Zn^{+2} and Cd^{+2} activation and Cd^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity *in vitro*. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

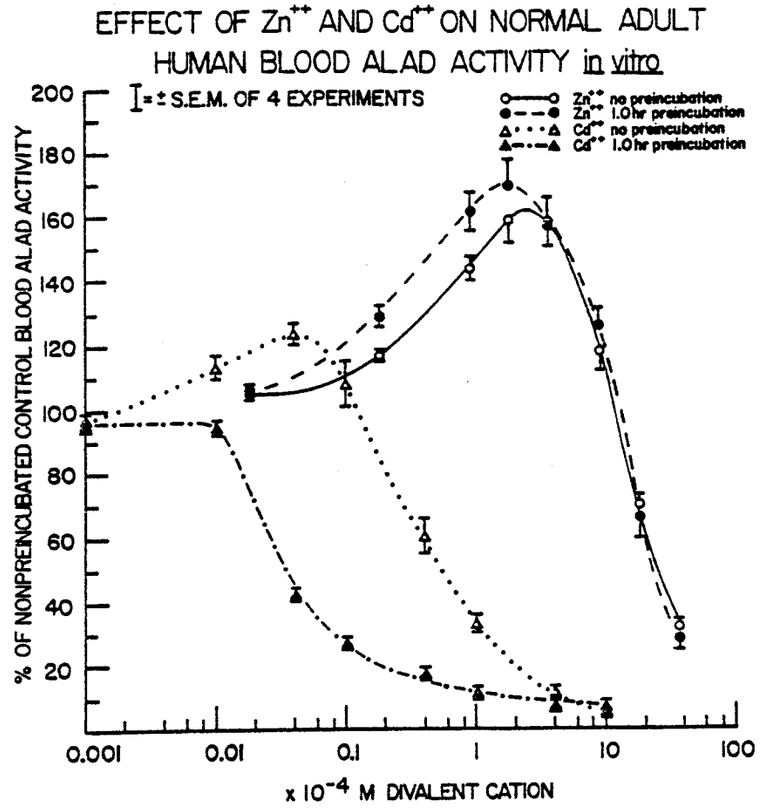

FIGURE 21

FIGURE 22. The effect of 0.5, 1.0, 2.0, and 4.0 hour preincubation with Pb^{+2} on Pb^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

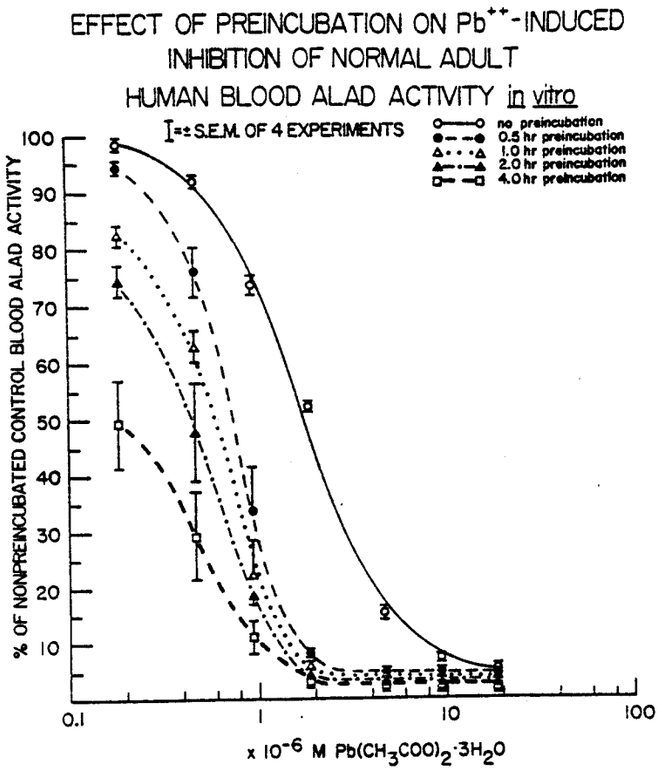

FIGURE 22

FIGURE 23. The effect of 0.5, 1.0, 2.0, and 4.0 hour pre-incubation with Pb^{+2} on the inhibition of normal adult human erythrocytic ALAD induced by various concentrations of Pb^{+2} in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

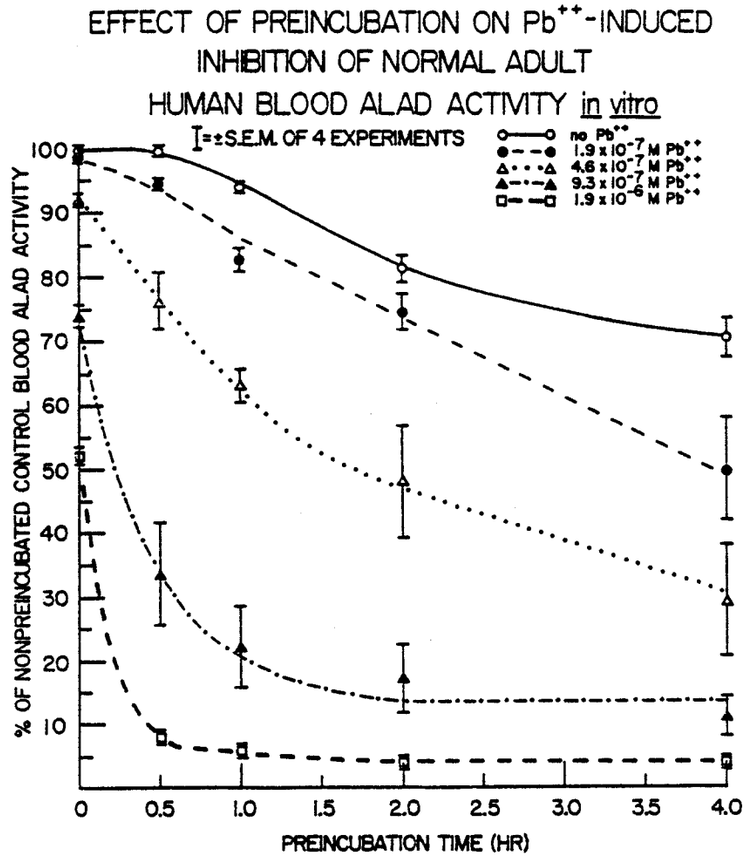

FIGURE 23

FIGURE 24. The effect of 0.5, 1.0, 2.0, and 4.0 hour pre-incubation with Cd^{+2} on Cd^{+2}-induced stimulation and Cd^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

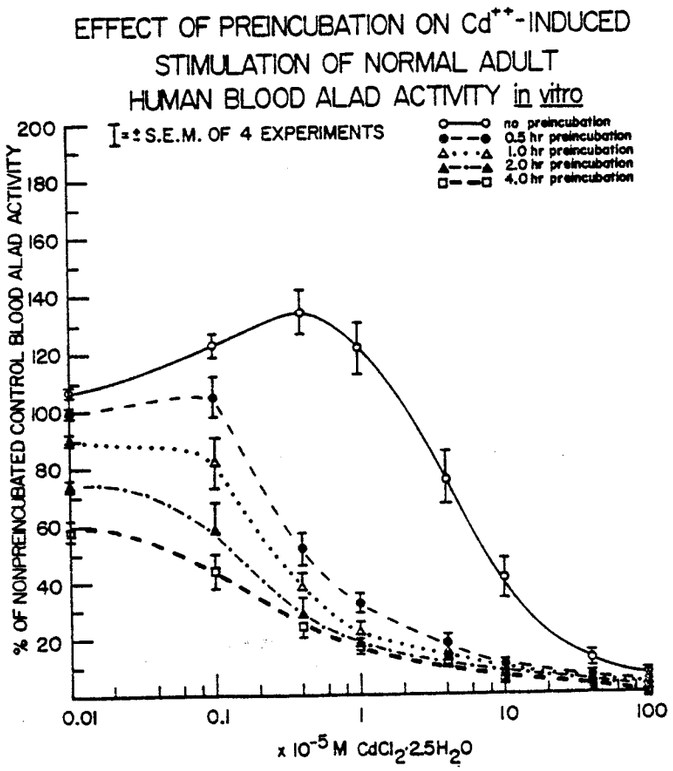

FIGURE 24

FIGURE 25. The effect of 0.5, 1.0, 2.0, and 4.0 hour pre-incubation with Cd^{+2} on the stimulation and inhibition of normal adult human erythrocytic ALAD induced by various concentrations of Cd^{+2} *in vitro*. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

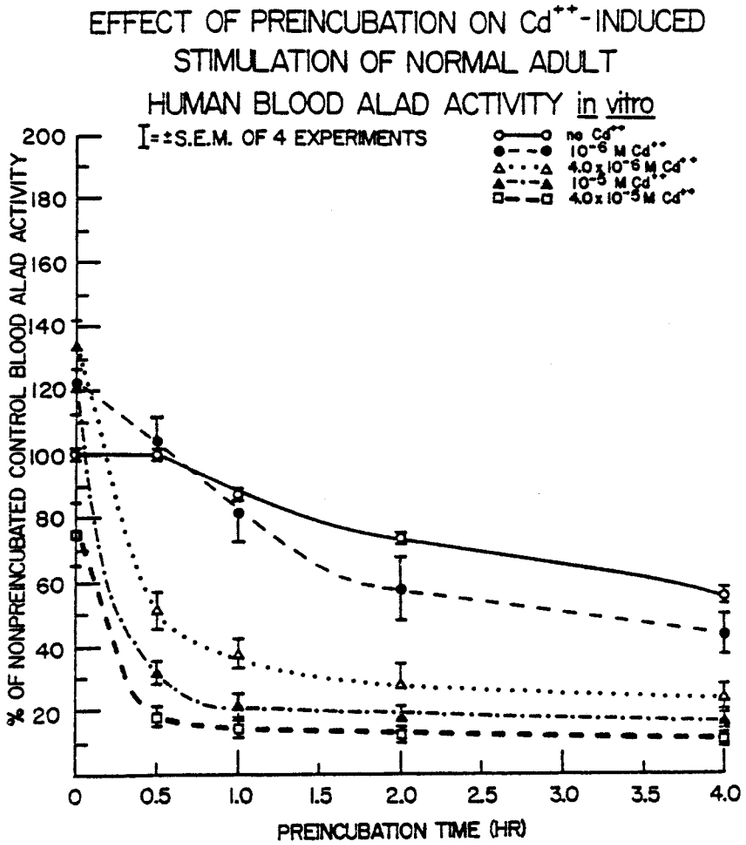

FIGURE 25

FIGURE 26. The effect of 0.5, 1.0, 2.0, and 4.0 hour pre-incubation with Zn^{+2} on Zn^{+2}-induced stimulation of normal adult human erythrocytic ALAD activity *in vitro*. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

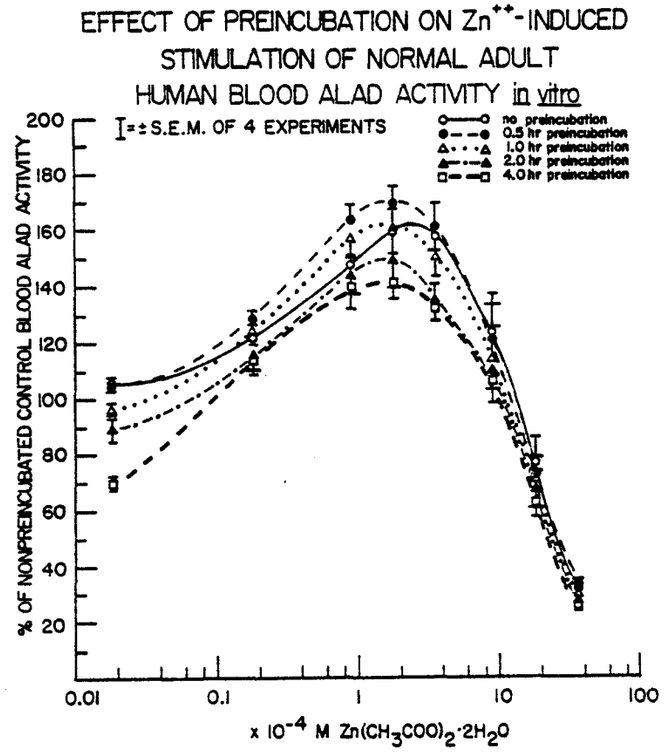

FIGURE 26

FIGURE 27. The effect of 0.5, 1.0, 2.0, and 4.0 hour pre-incubation with Zn^{+2} on the stimulation of normal adult human erythrocytic ALAD induced by various concentrations of Zn^{+2} <u>in vitro</u>. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

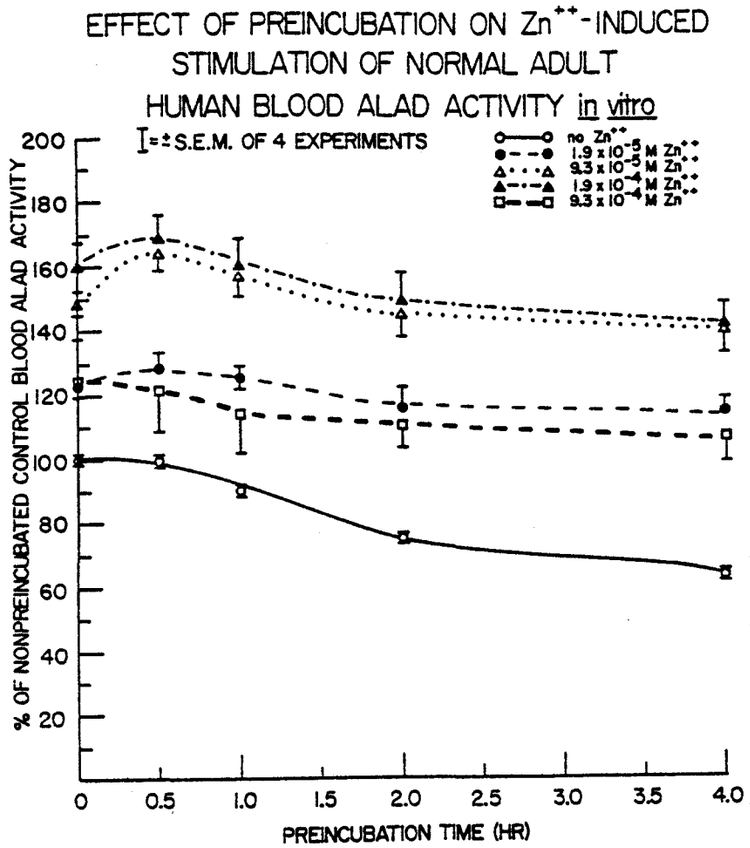

FIGURE 27

FIGURE 28. The effect of Pb^{+2}, Zn^{+2}, Cd^{+2}, and Hg^{+2} on normal adult human erythrocytic ALAD activity when added in vitro without, before, and after a 1.0 hour preincubation of the hemolysate. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

THE EFFECT OF Pb^{+2}, Zn^{+2}, Cd^{+2}, AND Hg^{+2} ON NORMAL ADULT HUMAN BLOOD ALAD ACTIVITY WHEN ADDED in vitro WITHOUT, BEFORE, AND AFTER A 1 HR. PREINCUBATION OF THE HEMOLYSATE

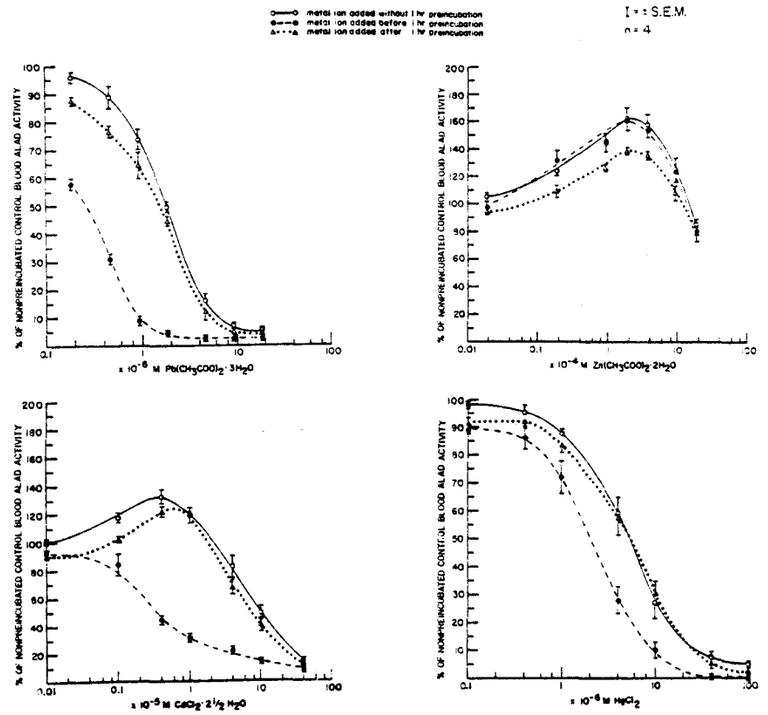

FIGURE 28

FIGURE 29. The effect of various concentrations of Zn^{+2} added before and after preincubation on preincubation ID_{50} Pb^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

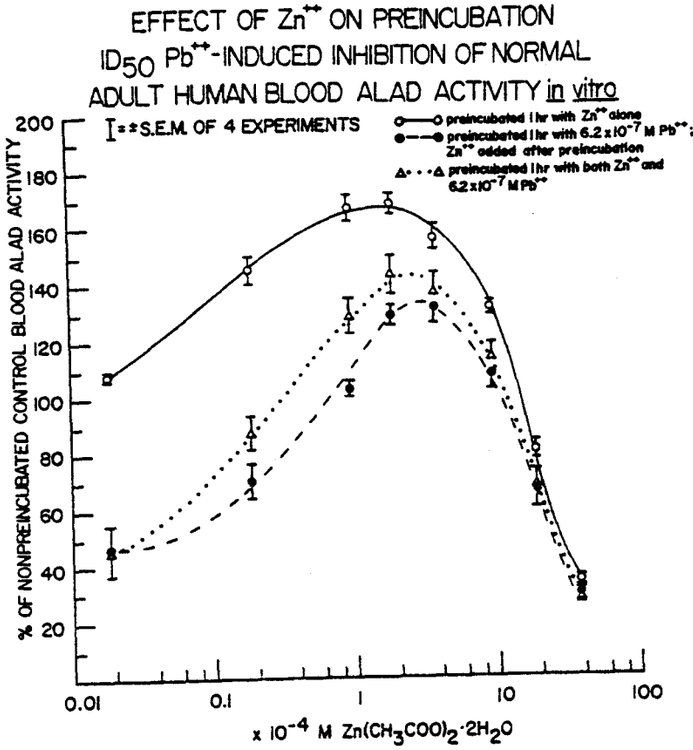

FIGURE 29

FIGURE 30. The effect of preincubation with various concentrations of Zn^{+2} on preincubation and nonpreincubation ID_{50} Pb^{+2}-induced inhibition of normal adult human erythrocytic ALAD activity in vitro. Each point represents the mean ± S.E.M. of results obtained for 4 individuals.

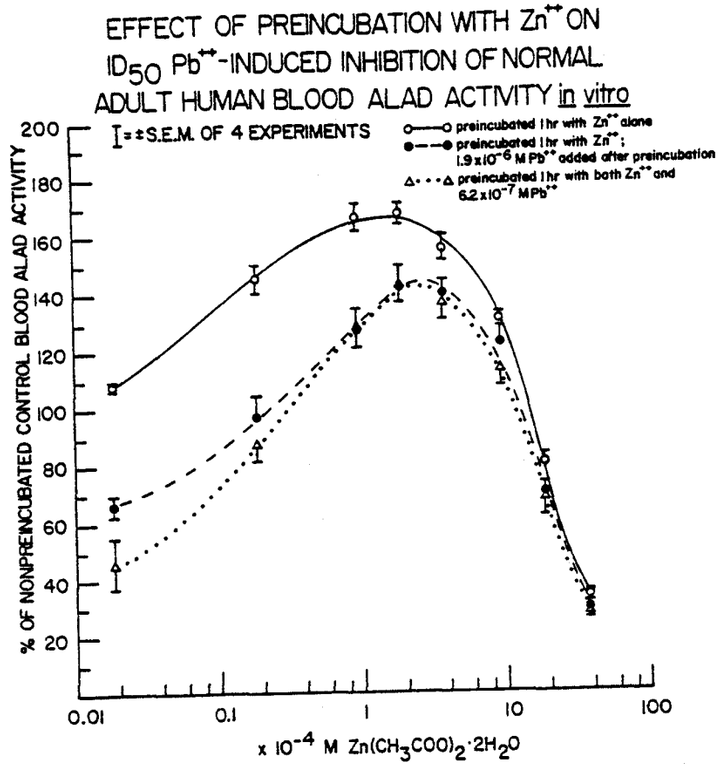

FIGURE 30

CHAPTER VIII

TABLES

TABLE 1. The reference values (Mean ± 2 S.D.) for the human erythrocytic ALAD activity of normal, nonmedicated adult subjects. The significance of a difference between sample means was evaluated by the two-tail non-paired t-test.

TABLE 1

REFERENCE VALUES (MEAN ± 2 SD) FOR THE HUMAN ERYTHROCYTIC ALAD ACTIVITY OF NORMAL SUBJECTS

	Number of patients	Age	Hemoglobin	Hematocrit	Rct%	Erythrocytic ALAD (mU/ml RBC)
Male	57	36.3 ± 23.4	15.4 ± 1.9	45.5 ± 5.6	1.2 ± 1.0	44.2 ± 16.5
Female	49	37.9 ± 29.6	13.6 ± 1.9	40.9 ± 6.0	1.2 ± 1.2	48.2 ± 17.3
Significance level (P)	--	N.S.	<0.001	<0.001	N.S.	<0.01

TABLE 2. The physical and chemical properties of metal ions and their effect on normal adult human erythrocytic ALAD activity in vitro.

TABLE 2

PHYSICAL AND CHEMICAL PROPERTIES OF METAL IONS AND THEIR EFFECT ON HUMAN ERYTHROCYTIC DELTA-AMINOLEVULINIC ACID DEHYDRATASE (ALAD) ACTIVITY in vitro

METAL	PERIODIC TABLE GROUP	OXIDATION STATE	VALENCE ELECTRONIC CONFIG.	CHARACTERISTIC COORDINATION NUMBER	COORDINATION GEOMETRY	PEARSON HSAB CLASSIF. OF LEWIS ACIDS	ALAD ID_{50}	ALAD SD_{100}
Na	Ia	+1	(Ne)	-	-	HA	> 10^{-3}M	-
Ag	Ib	+1	(Kr) $4d^{10}$	2	sp linear	SA	2 x 10^{-6}M	-
Mg	IIa	+2	(Ne)	-	-	HA	> 10^{-3}M	-
Zn	IIb	+2	(Ar) $3d^{10}$	4	sp^3 tetrahedral	Borderline Acid	3 x 10^{-3}M	2 x 10^{-4}M
Cd	IIb	+2	(Kr) $4d^{10}$	4	sp^3 tetrahedral	SA	1 x 10^{-4}M	4 x 10^{-6}M
Hg	IIb	+2	(Xe) $4f^{14}5d^{10}$	2	sp linear	SA	6 x 10^{-6}M	-
Al	III	+3	(Ne)	-	-	HA	1 x 10^{-2}M	-
Ga	III	+3	(Ar) $3d^{10}$	4	sp^3 tetrahedral	HA	7 x 10^{-4}M	-
In	III	+3	(Kr) $4d^{10}$	4	sp^3 tetrahedral	HA	7 x 10^{-4}M	-
Sn	IV	+4	(Kr) $4d^{10}$	4	sp^3 tetrahedral	HA	6 x 10^{-4}M	-
Sn	IV	+2	(Kr) $5s^2 4d^{10}$	3	sp^3 ?-tetrahedral	Borderline Acid	4 x 10^{-5}M	-
Pb	IV	+2	(Xe) $6s^2 4f^{14} 5d^{10}$	3	sp^3 ?-tetrahedral	Borderline Acid	2 x 10^{-6}M	-
Mn	VIIb	+2	(Ar) $3d^5$	6	d^2sp^3 octahedral	HA	> 10^{-3}M	-
Cu	Ib	+2	(Ar) $3d^9$	4	dsp^2 square planar	Borderline Acid	2 x 10^{-6}M	-

CHAPTER IX

BIBLIOGRAPHY

CHAPTER IX

BIBLIOGRAPHY

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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